ISOPRENYLATION OF TESTICULAR CELL PROTEINS IN RAT SEMINIFEROUS EPITHELIUM

By

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This dissertation is dedicated to my parents, Barbara and John Dugan, and to my fiance, Dwight Pitcairn.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

BSA	bovine serum albumin
Ci	curie
cm	centimeter
°C	degree centigrade
DNase	deoxyribonuclease
dpm	disintegrations per minute
DTT	dithiothreitol
IPP	isopentenyl diphosphate
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
h	hour
HMG CoA	3-hydroxy-3-methyl-glutaryl coenzyme A
M	molar
μ	micron
μg	microgram
μl	microliter
μM	micromolar
μmole	micromole
mM	millimolar
mmole	millimole
nmole	nanomole
PBS	phosphate buffered saline

PMSF	phenylmethylsulfonyl fluoride
PFT	protein farnesyl transferase
PGGT-I	protein geranylgeranyl transferase-I
PGGT-II	protein geranylgeranyl transferase-II
TLC	thin layer chromatography
Tris	Tris-(hydroxymethyl)aminomethane

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

THE ISOPRENYLATION OF TESTICULAR CELL PROTEINS IN THE SEMINIFEROUS EPITHELIUM

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The isoprenylation of testicular cell proteins was examined in prepuberal rat seminiferous epithelium, spermatogenic cells, and in isolated pachytene spermatocytes (PS, pre-meiotic) and round spermatids (RT, post-meiotic). Studies show protein farnesyl transferase (PFT) activity in the testes and spermatogenic cells of 23-day-old rats to be 2- to 3-fold higher than in 9-day-old animals and higher than in older animals. PS and RT from adult rats had the same level of PFT activity. PFT activity in PS from 23-day-old rats was 2 fold higher than adult PS and RT, yet the whole spermatogenic cell population from the same 23-day-old rats showed even higher activity (4 fold). PFT activity assayed in pooled Sta Put fractions of 23-day-old spermatogenic cells was highest in the fraction containing

cells slightly smaller than pachytene spermatocytes, possibly including secondary spermatocytes. Although the PS are the most prevalent cell type at 23 days, they alone do not appear to be responsible for the peak in PFT activity. The seminiferous epithelium from different aged animals was labelled with [^{3}H]-mevalonate (MVA) and the protein bound polyprenol analyzed following methyl iodide treatment. TLC revealed geranylgeraniol (GG) and farnesol (F) as the only products with GG/F ratios that decrease approximately 1.5 fold from 9 to 17 days with little change in older animals. Polyprenol analysis of PS and RT from adult animals showed GG/F ratios of 3.3 and 0.8, respectively, whereas the whole spermatogenic cell population had a ratio of 1.9. Two-dimensional PAGE of [^{3}H]-MVA labelled proteins showed age dependent changes of the levels of prenylation of at least 14 proteins. These results show cell type differences in geranylgeranylation and farnesylation and suggest age and cell dependent changes in protein acceptors in rat testes.

CHAPTER I
BACKGROUND, SIGNIFICANCE, AND OBJECTIVES

Background

With the advent of post-translational modification of proteins with isoprenoids and the implications of a role for this feature in cell cycling came the hypothesis that protein prenylation was involved in development. This work describes changing features of protein prenylation during spermatogenesis.

Spermatogenesis

Spermatogenesis entails many morphological and biochemical changes for the differentiating germ cells within the seminiferous tubules of the testes. Each germ cell undergoes three main phases of spermatogenesis, beginning with spermatogonial renewal and proliferation. This phase involves six mitotic divisions. Type A spermatogonia divide and differentiate into intermediate spermatogonia and type B spermatogonia. The last mitosis of type B spermatogonia results in the formation of preleptotene primary spermatocytes. These cells migrate from the basal compartment to the adluminal side of the tight

junctions of the epithelium formed by the Sertoli cells, and replicate their DNA before entering the second phase of spermatogenesis, meiosis. Distinct stages of meiotic prophase are termed leptotene, zygotene, pachytene, diplotene, and finally diakinesis to form secondary spermatocytes. Immediately following diakinesis, a second meiotic division occurs forming the haploid spermatids. Spermiogenesis, the final phase of spermatogenesis, consists of complex morphological transformations of the nucleus and acrosome. At spermiation, the cytoplasm separates from the flagellum, creating a residual body which is taken up by the Sertoli cell in phagocytosis. The late spermatid is released into the lumen of the seminiferous tubule.

The stages of spermatogenesis follow one another in a regular fashion along the seminiferous tubule. At any point in the tubule, there are well defined cell associations with one to two generations of spermatogonia, spermatocytes, and spermatids in each stage. Leblond and Clermont defined 14 separate stages of distinct durations that cycle in the rat (1). Man has six stages, whereas the monkey and the mouse have 12. The time interval between the reappearance of one particular cell association stage is the cycle of the seminiferous epithelium, and the succession of the 14 stages along the length of the tubule is the wave. The wave repeats itself along the length of an individual tubule in a sequence of about 12 waves per tubule. In the postnatal rat,

the cycle begins with gonocytes appearing at day four of age and ends with the first spermatozoa at day 45. In each segment of the tubule the cycle repeats itself every 12 days to provide a continuous wave of spermatozoa production throughout adult life.

Sertoli Cells

The seminiferous epithelium consists of Sertoli cells and germ cells organized in highly structured tubules by the surrounding peritubular cells. In 1865, the Italian physiologist Enrico Sertoli first identified the somatic component of the seminiferous epithelium. He described the cells as tall and columnar, extending perpendicularly from the basement membrane to the lumen of the seminiferous tubule, and enveloping the many associated germ cells (2). These Sertoli cells perform a nurturing role for the spermatocytes, analogous to the granulosa cells function during maturation of the oocyte in the ovary. Tight junctions, formed between the Sertoli cells, create a blood-testes barrier that isolates and immunologically protects the germ cells from the general circulation. The transport and function of nutrients and hormones from the circulation are mediated by the Sertoli cells in maintenance of germ cell development. The Sertoli cells are the primary targets for pituitary follicle-stimulating hormone (FSH) and androgens. The androgens are secreted by the Leydig cells,

which are strategically positioned in the testicular interstitial tissue, between tubules. Since FSH and testosterone are known to regulate spermatogenesis, the importance of Sertoli cells in mediating and regulating the process of sperm production has been emphasized by several investigators (3-6).

Cyclic Activity

In support of the controlling influence Sertoli cells impose on spermatogenesis are the variations in Sertoli cell biochemical activities observed in different stages of spermatogenesis. There are also cyclic morphological changes in the shape of the nuclei of the Sertoli cells and in the abundance and distribution of lipid droplets (7). Recent morphometric analyses have shown cyclic decreases in Sertoli cell vessicle volume density and increases in the endoplasmic reticulum volume density (8). These observations are indicative of active and variable cellular activities. The germ cells also exhibit stage-specific cyclic changes with drastic morphological alterations in size, density, nuclear arrangement, and acrosome development (9).

The constituent protein changes in spermatogenic cells reflect the changes found in spermatogenic cell mRNA populations with differential gene expression during spermatogenesis (10). For example, the expression of the cellular protooncogenes N-,Ki-, and Ha-ras has been found to

be differentiation specific during mouse germ cell development (11,12). Stage specific expression of these protooncogenes suggest that these genes normally participate in the differentiative process. N-ras mRNA is specifically detected in postmeiotic early spermatids, whereas the Ki-ras transcript is expressed mainly in the meiotic pachytene spermatocytes. On the other hand, Ha-ras transcript is reported to be low in both meiotic and postmeiotic cells. The absence of transcripts of N-ras in mutant sterile mice (Sl/Sl^d) which lack germ cells, yet its presence in the mutant sterile quaking (qk) mice which have spermatids, supports the idea that these protooncogenes and their prenylated protein products play a role in germ cell development. Another example of differential gene expression during spermatogenesis is a testis-specific transcript of rat farnesyl diphosphate synthase that was detected by RNA blot analysis and in situ hybridization (13). This transcript is exclusively expressed in round spermatids at stages 7 to 8 in the seminiferous epithelium. Mouse protamine genes are also solely expressed in haploid spermatids (14,15).

Extensive chromosome activity occurs during meiosis and spermiogenesis as described by Monesi (16). RNA synthesis undergoes a rapid increase in synthesis to a peak in late pachytene spermatocytes, followed by a decline in diplotene and during diakinesis. The RNA molecules synthesized in

meiosis, "meiotic RNA" remain associated with the chromosomes until diakinesis when they are rapidly released into the cytoplasm. This is the primary RNA present in the spermatid cytoplasm and its protein products most likely direct differentiation in spermiogenesis since this time is also characterized by genetic inactivation and arrest of nuclear protein synthesis.

The cell types of the seminiferous epithelium exhibit high metabolic activity with increased protein production and secretion. Enzyme activities of the Sertoli cells and the spermatogenic cells have been shown to vary depending on the stage of the cycle of the seminiferous epithelium. For example, both acid phosphatase and thiamine pyrophosphatase activities were found to peak in stages VII and VIII, with little activity in stages IX through II (17). The Sertoli cells secrete both testes-specific and serum proteins (18), many of which have been found to be secreted in a stage-specific, cyclic manner. For example, androgen binding protein (ABP) (6), plasminogen activator (19), and testicular transferrin (20) show fluctuations in secretion rate with specific stages of spermatogenesis. Fucosylation during the post-translational modification of proteins, particularly membrane glycoproteins, are continually changing in the differentiating germ cells (21). Differences in a host of cell specific proteins in the different stages and different cell types has been noted. For example, the

somatic cells including Sertoli cells contain lamins A, B, and C, whereas the spermatogenic cells have lamin B, but do not reveal immunologically reactive lamins A and C (22).

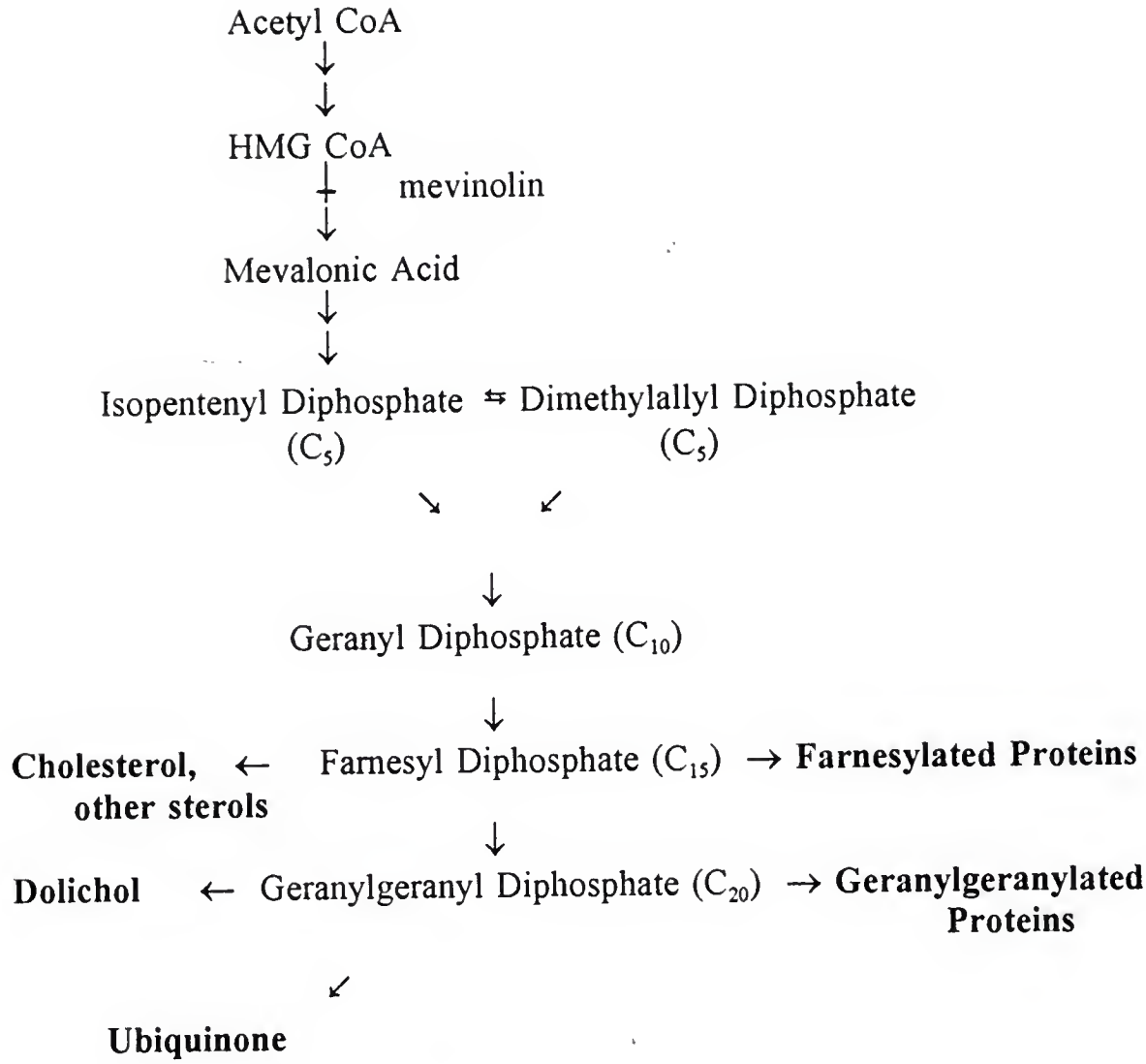
Enzymes in isoprenoid biosynthesis have also been shown to change. Potter et al. observed increased rates of [^{14}C]-acetate incorporation into cholesterol in pachytene stages of spermatogenesis and high rates of incorporation into dolichol in differentiating spermatocytes, particularly the pachytene spermatocytes (23). Experiments performed in this lab have shown a cyclic change in the specific enzymatic activity of 2,3-dehydrodolichyl diphosphate synthase in enriched spermatogenic and Sertoli cell populations (24, 25). The highest activity occurs in 23 day old rats and is also attributed to the appearance of pachytene spermatocytes. The fact that spermatogenesis entails cytochemical changes in gene expression, enzyme activities, protein secretion, protein distribution and protein modification is therefore well documented.

Isoprenoid Biosynthesis

The rate-controlling enzyme for isoprenoid biosynthesis is 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (see Fig. 1-1). The product of this enzyme reaction is mevalonate, the precursor of many isoprenoid groups. Mevalonate is phosphorylated and decarboxylated to form isopentenyl diphosphate (IPP), a C_5 isoprene intermediate.

Figure 1-1. The Isoprenoid Biosynthetic Pathway

HMG CoA Reductase is the rate-limiting enzyme for the production of isoprenoids. It is inhibited by the pharmacological agent mevinolin.


Acetyl CoA
HMG CoA
mevinolin
Mevalonic Acid
Isopentenyl Diphosphate ⇆ Dimethylallyl Diphosphate
(C5)
(C5)
Geranyl Diphosphate (C10)
Cholesterol, other sterols
Farnesyl Diphosphate (C15)
Farnesylated Proteins
Dolichol
Geranylgeranyl Diphosphate (C20)
Geranylgeranylated Proteins
Ubiquinone

Dimethylallyl diphosphate is synthesized by the isomerization of IPP and then combines with IPP through three steps, ionization, condensation, and elimination to form the C_{10} compound geranyl diphosphate. Similarly, IPP reacts with geranyl diphosphate to give the C_{15} product, farnesyl pyrophosphate (FPP), and with FPP to give the C_{20} product geranylgeranyl diphosphate (GGPP) (26, 27). FPP occupies a central position in the isoprenoid pathway for the formation of several end products of mevalonate metabolism including cholesterol, dolichol, and ubiquinone, each essential for normal cell function. Cholesterol is a membrane component, a bile acid precursor, as well as a steroid hormone precursor. Dolichol is a sugar carrier important in N-linked glycoprotein biosynthesis and ubiquinone is a necessary redox catalyst in electron transport. Other non-sterol mevalonate-derived products include isopentenyl adenosine, heme a, farnesylated and geranylgeranylated proteins. So far, the only known function for all-trans-GGPP in mammalian cells is the modification of proteins to form geranylgeranylated proteins. The synthesis of trans,trans,cis-GGPP as an intermediate in dolichol synthesis is membrane-associated (28, 29).

The synthesis of mevalonate is strictly regulated in the cell in order to maintain isoprenoid availability without over-producing sterols (30). HMGCoA synthase, HMGCoA reductase, farnesyl diphosphate synthase (prenyl

transferse), and squalene synthase are feedback regulated transcriptionally by cholesterol, the main metabolite of mevalonate (30-33). Cholesterol also inhibits expression of the LDL receptor gene in order to limit the contribution of cholesterol from plasma LDL to the cellular cholesterol. HMGCoA synthesis is further inhibited by a non-sterol mevalonate-derived isoprenoid at both translational and post-translational levels (34-36). The latter is achieved by increasing reductase degradation. Brown and Goldstein have speculated that the responsible isoprenoid product is a prenylated protein (37), although other acute processes may be involved (38). It is also speculated that the non-sterol pathways have higher affinities for mevalonate metabolites (e.g. FPP) than the sterol pathway, which would display yet another measure of control to ensure synthesis of the non-sterol isoprenoid products especially when mevalonate is limiting (30).

Inhibition of HMG CoA reductase and hence the synthesis of mevalonate prevents protein prenylation and blocks cell growth (38-44). The drugs compactin and mevinolin are competitive inhibitors of HMGCoA reductase (45) and have been shown to halt cell entry into the S phase of the cell cycle (30, 36, 46). Subsequent addition of mevalonate restores growth to the drug treated cell cultures. The addition of serum lipoproteins or cholesterol can reduce the amount of mevalonate required, but not replace it. This

suggested a requirement for a separate, non-sterol product of mevalonate for cell cycling (47).

In 1984, Schmidt et al. first showed post-translational incorporation of isoprenoids into cellular proteins using radiolabeled mevalonate (48). After mevinolin treatment, the addition of radiolabelled mevalonate resulted in enhanced labelling of proteins (49). Sinensky and Logel then suggested that a halt in DNA replication in mevalonate starved Mev-1 cells was due to the failure to isoprenylate proteins (50). Since then, much emphasis has been focused on identifying the isoprenylated proteins, the nature of isoprene attachment and the function of the modification.

Protein Isoprenylation

The secreted fungal mating factors were the first isoprenylated polypeptides identified, and the isoprenoid moiety was identified as farnesyl (see Fig. 1-1). The first organism discovered to contain a prenylated peptide was Rhodosporidium toruloides (51); however, the mechanism of farnesylation has been studied in particular in the mating a-factor of Saccharomyces cerevisiae (52). Immediately after translation, the mating a-factor peptide undergoes several chemical modifications which are similar to those found in the $p21^{ras}$ protein processing. These events involve attachment of a farnesyl group to the thiol of the cysteine, four amino acid residues from the carboxy terminus.

Subsequent proteolytic cleavage removes the three carboxy terminal amino acids and leaves the farnesylated cysteine at the carboxy terminus, which is then methylated. This series of post-translational modifications is necessary for secretion of the yeast a-factor (42). The carboxy terminal sequence termed the CaaX motif (C is cysteine, a is usually an aliphatic amino acid, and X is variable) has been found in many other isoprenylated proteins, including the ras family of guanine nucleotide binding proteins (G-proteins), that share the same processing events (42). The term CaaX will continue to be used here; however, it has been reported that the aliphatic amino acid is not a strict requirement (especially for the amino acid adjacent to the cysteine) (53, 54) as was originally suggested. Attachment of the isoprenoid moiety is required for membrane association and biological function of the ras proteins (55). Several isoprenylated GTP-binding proteins, distinct from the ras family, have been identified in MEL cells by Maltese et al. (56). The nuclear envelope protein, lamin B in Swiss 3T3 cells, HeLa cells, CHO cells, and MEL cells also contains a farnesyl group (41, 49). The farnesyl group of prelamin A has been implicated to function in nuclear localization and target assembly of this protein to the nuclear envelope (57). Prelamin A subsequently loses the isoprenoid moiety concurrently with a 2kD mass reduction (40, 44). Other proteins have also been ascertained to contain the farnesyl

moiety. These include the γ subunit of transducin (58) and the γ subunit of photoreceptor GTP-binding protein (59). It has been determined that farnesylation of the latter is necessary for GTP binding. Also, full enzymatic activity of rhodopsin kinase was found to require farnesylation and α-carboxyl methylation (60).

It soon became apparent, however, that a majority of the cellular prenylated proteins were linked with geranylgeraniol (61, 62). The γ subunits of trimeric G proteins (63-65) and many low molecular weight ras-like GTP-binding proteins were found to be geranylgeranylated. The low molecular weight G proteins include G25K (Gp) (66), rac 1, rac 2, and ral A (53), rap 1A (Krev-1) (67), and smg p25A. The latter was found to be modified with two geranylgeranyl moieties and a methyl ester (68). Many of the low molecular weight GTP-binding ras-like proteins that are geranylgeranylated have carboxy terminal sequences that vary from the CaaX motif (i.e. XXCC, CCXX, or XCXC). The rab family of proteins, including rab 1B, rab 2, rab 5, and rab 6 are also geranylgeranylated (69). The rab proteins have been suggested to participate in the movement of intracellular vessicles (70). These proteins are clearly required in nuclear assembly, signal transduction, and vessicle transport; which are processes necessary for normal cell function as well as proliferation.

Protein Prenyl Transferases

Three enzymes have been purified that recognize specific CaaX or other cysteine containing sequences which are targets for prenylation. The protein farnesyl transferase (PFT) was purified to homogeneity from rat (71) or bovine brain (72) as an α/ß heterodimer. Each subunit of the rat brain enzyme have been subsequently cloned by the Brown and Goldstein group (73, 74). The molecular masses of the α and ß subunits are 47 kDa and 45 kDa, respectively. Cross-linking experiments implicate the ß subunit in functionally binding the protein substrate (75). PFT preferentially farnesylates proteins or peptides with the CaaX sequence where X is Ala, Ser, Gln, Cys, or Met, but most commonly Ser or Met (54, 72, 76-78). Protein geranylgeranyl transferase I (PGGT-I) purified from bovine brain is an α/ß heterodimer (79) which geranylgeranylates proteins with the CaaX sequence where X is Leu (72, 76-78, 80). Selective prenylation of CaaX tetrapeptides indicates that only the four terminal amino acids of the protein substrates are necessary determinants for prenylation by PFT and PGGT-I (78). Antibodies to the α subunit of rat brain PFT cross react with the α subunit of PGGT-I indicating that these prenyl transferases share a common subunit (75). Genetic studies in Saccharomyces cerevisiae also indicate a common genetic feature between PFT and PGGT-I since mutations in ram2 give decreased activity for both enzymes

(78, 81, 82). In contrast, mutations in dpr1/ram1 are specific for PFT, indicating the dpr1/ram1 gene is responsible for only PFT activity (78, 81). Correlatively, CDC43/CAL1 was found to be essential for PGGT-I and not PFT activity (81). Therefore, the yeast protein RAM2 is homologous to bovine PFT α, whereas RAM1 and CDC43/CAL1 share homology with the ß subunit of PFT and PGGT-I, respectively (83).

The yeast gene BET2 shows homology to RAM1 and CDC43 genes, but bet2 mutants can still prenylate proteins with the CaaX sequence. On the other hand, bet2 mutants do not prenylate the GGCC-containing YPT1 protein (83). A third enzyme, PGGT-II or Rab geranylgeranyl transferase, comprised in part of the BET2 protein, geranylgeranylates proteins with carboxy terminal sequences other than CaaX. These sequences are CXC (such as rab 3A, rab 4, and rab 6), GGCC (such as rab 1A, rab 1B, and rab 2), and CCXX (such as rab 5) (68, 84-87). At least the rab 3A protein has been shown to contain two geranylgeranyl moieties (68). However, peptides of the carboxyl terminus of these proteins do not compete for protein geranylgeranylation, indicating the interaction between PGGT-II and its protein substrate is more complex than that exhibited by PFT and PGGT-I for their CaaX subsrates (69, 72, 84). PGGT-II activity from rat brain cytosol separates into two components, A and B (86). Component B consists of two polypeptides of 60 and 38 kDa

whose amino acid sequences resemble in part those of the α and ß subunits of ras PFT. Component A is a 95 kDa protein with no counterpart in PFT. Brown and Goldstein have speculated that there are a family of A components that recognize the variable protein substrates and are tissue specific as well (88).

Significance

Earlier work showed that enzymes and intermediates of the isoprenoid biosynthetic pathway changed during differentiation. For example, the availability of the isoprenoid dolichyl phosphate has been found to be rate-limiting for synthesis of glycoproteins and a possible regulatory component in developmental processes (25, 89-91). The activity of one of the enzymes required for dolichyl phosphate synthesis, the prenyl transferase 2,3-dehydrodolichyl diphosphate synthase, has been shown to cycle in coordination with increased concentration of dolichyl phosphate during spermatogenesis (25). Furthermore, Kabakoff et al. report the level of dolichyl phosphate available for N-linked protein glycosylation correlates with cell proliferation during mevalonate depletion and repletion (92). They suggest the synthesis of dolichyl phosphate may be rate-limiting for cell proliferation, although they do not rule out the role of other isoprenoid products in

regulation of cell growth, such as prenylated proteins. Characterization of the prenyl transferases involved in isoprenoid attachment to proteins has recently become of interest since it has been speculated that this post-translational modification is necessary for cell proliferation, yet has not been studied in a developmental system. As described above the expression of the cellular protooncogenes N-, Ki-, and Ha-ras is stage specific suggesting that these genes and their prenylated protein products normally participate in the differentiative process (11, 12). A recent report, that may have significance in a developmental system, described cell cycle dependent changes in prenylated proteins in HepG2 cells (93). Increased protein prenylation occurred at the G1/S interface of the cell cycle, precisely where cell cycling ceased following mevalonate deprivation. Maltese and Sheridan, however, suggested that the profile of mevalonate labelled proteins in a given cell line is not altered by malignant transformation (49). Since the isoprenoid attachment to the ras family of proteins is required for ras-mediated transformation (55) and prenylated proteins pertain to the non-sterol mevalonate products necessary for cell cycling (47, 50), one can speculate that protein prenylation is required in the mitotic and meiotic events during the differentiative process of spermatogenesis. The recent developments on protein prenylation prompted me to examine

changes in prenyl transferase activities, modes of protein prenylation, and the types of proteins prenylated during spermatogenesis.

Objectives

The main objectives of this dissertation are to study the activities of the protein prenyl transferases and the in vivo labelling of proteins with polyprenols during spermatogenesis in the rat and correlate the findings with known cellular transformations.

Specific objectives are the following;

A. Optimize the assay conditions for protein farnesyl transferase (PFT) and protein geranylgeranyl transferase-I (PGGT-I) in cytosolic fractions from testicular cells.

B. Determine the specific activities for each enzyme in the cytosols of the testes of different aged rats.

C. Determine the specific enzymatic activities in the cytosols of different spermatogenic cell types (particularly the pachytene spermatocytes and the round spermatids) and in seminiferous epithelium from rat testes.

D. Optimize labelling of proteins of the seminiferous epithelium with [^{3}H]-mevalonic acid.

E. Analyze the polyprenols liberated by methyl iodide treatment of labelled proteins in the seminiferous

epithelium of rats of different ages and in different spermatogenic cell types.

F. Analyze selected labelled proteins of the seminiferous epithelium from rats of different ages and from different spermatogenic cell types by one-dimensional and two-dimensional gel electrophoresis.

Chapter II of this dissertation describes the studies toward fulfillment of objectives A through C. Chapter III describes the studies toward achievement of objectives D through F.

CHAPTER II
OPTIMIZATION OF ASSAYS FOR PROTEIN FARNESYL TRANSFERASE AND PROTEIN GERANYLGERANYL TRANSFERASE AND THEIR ANALYSIS DURING SPERMATOGENESIS

Introduction

Differentiation-dependent changes in protein prenyl transferases have not been described in any system. Spermatogenesis is a well described process of differentiation which we have chosen as our model system. In order to determine the capacity for testicular cells to prenylate proteins, the enzymatic activities of PFT and PGGT-I in rat testes were first characterized. These activities have been localized by others to the cytosol in testes and other mammalian tissues (41, 43, 48, 71, 76-78, 80, 82, 94-96). The highest levels of PFT activity have been described in rat and pig brain (94,95) but the levels of PFT and PGGT-I in testes has not been described. Chen et al. (93) have reported a large amount of PFT α subunit mRNA in the rat testes, but only low levels of PFT ß subunit mRNA have been described (74). Age dependent changes in PGGT-I activity have been reported in rat brain, liver, kidney and heart where the activity increased from birth and reached a plateaue at about 20 days of age (96).

The methods for the PFT assay were first described by Schmidt et al. (48) and Farnsworth et al. (41). The PGGT-I assays have been described by Seabra et al. (76), Yokoyama et al. (77), Yoshida et al. (78), and Joly et al. (96). These procedures have been modified, as described below, to optimize the analysis of protein prenylation in the soluble fraction of testicular samples obtained at different stages of spermatogenesis. This work describes the use of radiolabelled allylic diphosphates with either unmodified recombinant protein or biotinylated peptides to detect activity.

This chapter shows 1) optimal parameters for assaying testicular PFT and PGGT-I; 2) separate activities of these closely related enzymes in the same cytosolic preparation; 3) age-dependent changes in the levels of PFT activity in whole, decapsulated testicular cytosols and similar age-dependent changes in the levels of both PFT and PGGT-I activity in the cytosols of spermatogenic cells; and 4) comparisons of PFT and PGGT-I activities in different spermatogenic cell types. The age-dependent changes for both activities in spermatogenic cells show a 2-3 fold increase in specific activity from 9 to 23 days of age and a similar decrease from days 23 to 35. The evaluation of enzyme specific activities in different spermatogenic cell populations of rat testes show similar activities in pachytene spermatocytes and round spermatids yet higher PFT

activity in a third cell population. Possible roles for regulation of these enzymes at 23 days and in the third cell population are discussed.

Materials and Methods

Materials

Recombinant $p21^{H\text{-}ras}$ (valine 12) was the generous gift of Burroughs-Wellcome Research Laboratories, Burroughs Wellcome Co., NC. Recombinant MBP-G23K, MBP-rab 1B, MBP-rab 5, and MBP-rab 6 were gifts of W.A. Maltese, Weis Research Center, Geisinger Clinic, Danville, PA. The peptides, biotinyl-Lys-Thr-Lys-Cys-Val-Ile-Ser (Bt-KTKCVIS) and N-acetyl-KTKCVIS were synthesized by the Protein Chemistry Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida. Biotinyl-Lys-Lys-Phe-Phe-Cys-Ala-Ile-Leu (Bt-KKFFCAIL) was generously provided by Dr. A. Joly, University of California Los Angles. [^{3}H]-Geranylgeranyl diphosphate (8-15 Ci/mmol) and [^{3}H]-farnesyl diphosphate (20 Ci/mmol) were obtained from Dupont-NEN or American Radiolabelled Chemicals, Inc. Other reagents and enzymes not otherwise described were obtained from Sigma Chemical Co.

Animal Groupings

In order to have sufficient data for statistical analysis in each experiment, at least two rats were used for

each prepuberal age group tested. Studies with younger rats required more animals. For example, experiments with rats aged 9-18 days, 19-26 days, and 27-40 days, required 8-12 rats, 5-8 rats, and 2-4 rats, respectively. The excised testes from each aged group were pooled together for enzyme assay or subsequent cell separation. The data presented in each figure are usually means determined by two or three experiments.

Isolation of Seminiferous Epithelium, Spermatogenic Cells, and Spermatozoa

Rats were lightly anesthetized with methoxyflurane, decapitated with a guillotine, and the testes excised. The testes were decapsulated by cutting and removing the tunica albuginea while the tubules were gently expressed. The spermatic artery was removed and the tissue placed in a 125 ml siliconized flask containing 50 ml of McCoy's medium. The tubules are washed by unit gravity settling and aspirating of the supernatant. The seminiferous epithelium was prepared from the decapsulated tubules by a modification of a two-step enzymatic method described by Romrell for the mouse (97). First, 30 ml of 1 mg/ml collagenase and 2 μg/ml deoxyribonuclease (DNase) in McCoy's medium at 37°C was added to the tubules; the flask was covered and placed in a 37°C water bath for 50 min, with swirling and shaking every 5 min. At the end of the incubation, the suspension was washed 3 times by unit gravity settling for 2-3 min in 3

changes of 50 ml McCoy's medium. Then 30 ml of 2 mg/ml trypsin and 2 μg/ml DNase in McCoy's medium was added and swirled every 5 min in a 37°C water bath for 15 min. The trypsin was immediately neutralized by the addition of equal units of trypsin inhibitor in 20 ml McCoy's media. The cells were then mechanically dispersed by pipeting gently 50 times with a flamed, siliconized pasteur pipet. The spermatogenic cells were isolated by filtering the cells through a 74μ nylon mesh (Small Parts, Inc.) to remove Sertoli cell clumps (98). The spermatogenic cells were washed in McCoy's media three times by centrifuging at 200 x g for 5 min. Spermatozoa were isolated from the epididymis by cutting the tissue 8 times followed by suspension in 20 ml of McCoy's media. The tissue was allowed to settle by unit gravity and the media containing the mature sperm was removed. All cellular populations were counted with a hemocytometer and the viability determined by trypan blue exclusion. The viability was always 95 to 98%.

Spermatogenic Cell Fractionation

The STA-PUT unit gravity procedure for separating spermatogenic cells was performed similarly to the process described by Romrell et al. (98). The whole procedure was carried out at 4°C. A diagram of the procedure is depicted in Appendix A. The sedimentation chamber was initially filled with 20 mls of McCoys medium. The cell suspension

containing 10^8 spermatogenic cells in 40 mls of 0.5% BSA (Sigma Fraction V, lot# 12H0182) in McCoys medium was introduced into the chamber at a flow rate of 10 ml/min, followed by a linear gradient of 2% to 4% BSA in McCoys medium (2200 ml total volume). Five minutes after loading the cell suspension, the flow rate was increased to 40 ml/min. Fractions (10 ml) containing the separated spermatogenic cells were collected at a rate of 10 ml/min starting 3 h after loading the cell suspension. Within five hours after introducing the cell suspension to the chamber, the cell collection was finished. Samples from each fraction were examined by Nomarski differential interference and phase contrast microscopy. The fractions consisting of early spermatids (stages I through VIII) and pachytene spermatocytes were pooled separately and washed three times with McCoys medium by centrifuging at 200xg for 10 min.

Subcellular Fractionation and Preparation of Cytosolic Enzyme

Cytosolic fractions were used as the enzyme source. Isolated spermatogenic cells or decapsulated testes from rats of different ages were suspended in 50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 1 mM PMSF, 10% glycerol, then disrupted in a Dounce homogenizer by twenty strokes with a type A pestle. Particulate material was removed by consecutive centrifugations at 9,000 and 100,000 x g, resulting in the cytosolic fraction. The microsomal fraction

was collected from the pellet of the 100,000 x g centrifugation. Protein was estimated by the Biorad Protein assay using bovine serum albumin as a standard.

Protein Prenyl Transferase Assay

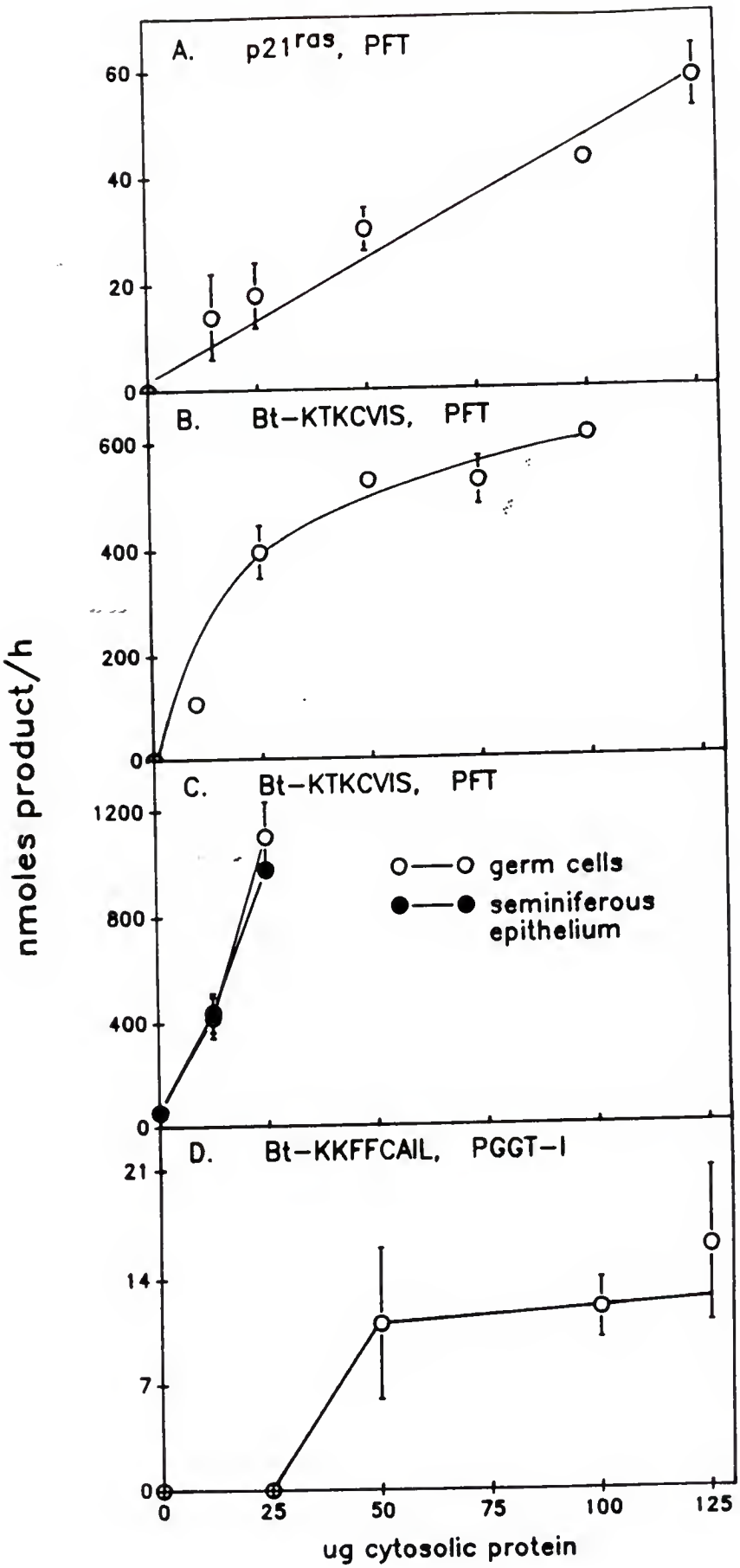
In the PFT assay, [^{3}H]-farnesyl diphosphate is incubated with either recombinant p21^{ras} or a biotinylated heptapeptide, Bt-KTKCVIS, whose sequence is similar to the carboxy terminal sequence of K-ras-2B (38) (the C-terminal Met was replaced with Ser for easier chemical synthesis). Similarly the PGGT-I assay was performed with [^{3}H]-geranylgeranyl diphosphate and recombinant chimeric proteins or a biotinylated octapeptide Bt-KKFFCAIL, whose sequence was designed after the carboxy terminal sequence of the γ subunit of bovine brain trimeric G proteins (99, 100). A high concentration of $MgCl_2$ (5mM) and a low concentration of $ZnCl_2$ (25 μM), which had been found by others to be stimulatory for both enzyme activities, were used in these experiments. PMSF was routinely added to the assay. Initially, pepstatin A and leupeptin were added to protect the protein from endogenous proteases and NaF was added to protect from endogenous phosphatases. However, there were no major changes in the extent of formation of radiolabelled enzymatic products when pepstatin A, leupeptin, and NaF were omitted from the assay.

The dependence of PFT and PGGT-I activities on cytosolic protein concentration with appropriate protein and peptide substrates is depicted in Figure 2-1,A,B,C, and D. In panel A and B, a linear response of PFT activity was detected for up to 125 μg protein with $p21^{ras}$ or for up to 25 μg protein for Bt-KTKCVIS, respectively. PGGT-I (Panel D) did not show a linear response when less than 50 μg of protein were used. On the other hand, higher protein concentrations did not lead to significant increases in activity. Therefore, 50 μg of protein was selected to assess PGGT-I activity. In the assays with the Bt-peptide and allylic substrate concentrations were saturating. This was not the case with the recombinant $p21^{ras}$ protein. Previous work with MOLT IV cells suggested that 11.4 μM $p21^{ras}$ is not saturating for PFT activity (101), which explains at least in part the lower levels of activity for the protein than with peptide substrates. Since the source of the recombinant protein was limiting, the biotinylated peptide was preferentially used for most of the experiments to follow.

Biotinylated peptides as prenyl acceptors. PFT and PGGT-I were assayed using biotinylated peptides Bt-KTKCVIS and BT-KKFFCAIL, respectively by a modification of the method of Farnesworth et al. (41). Each 25 μl reaction mixture contained 25-125 μg of cytosolic protein, 60 mM Tris-HCl, pH 7.5, 25 μM $ZnCl_2$, 5 mM $MgCl_2$, 2.0 mM dithiothreitol, 0.5 mM PMSF, 2% glycerol, 30 μM Bt-KTKCVIS

Figure 2-1. Cytosolic Protein Dependence for PFT and PGGT-I Activities.

Incubations contained various concentrations of cytosolic protein from decapsulated testes of a 70 day old rat (Panels A, B, and D) or germ cells and seminiferous epithelium from a 23 day old rat (Panel C). PFT activity was assayed as described in the methods with 11.4 μM recombinant $p21^{ras}$ (Panel A) or 30 μM Bt-KTKCVIS (Panels B and C). PGGT-I activity was assayed with 30 μM Bt-KKFFCAIL (Panel D). PFT assays were performed in duplicate and PGGT-I values were averaged from triplicate assays.


A. p21ras, PFT
B. Bt-KTKCVIS, PFT
C. Bt-KTKCVIS, PFT
germ cells
seminiferous epithelium
D. Bt-KKFFCAIL, PGGT-I
nmoles product/h
ug cytosolic protein
0
20
40
60
200
400
600
400
800
1200
7
14
21
25
50
75
100
125

or Bt-KKFFCAIL, and 0.4 μM [^{3}H]-t,t-farnesyl diphosphate (FPP) or 2.0 μM [^{3}H]-all trans-geranylgeranyl diphosphate (GGPP). Incubations were carried out at 37°C for 1 h followed by the addition of a suspension of avidin-agarose beads in 0.5 M NaCl, 0.02% NaN_3. The tubes were vortexed repeatedly during a 15 min interval, then the beads were washed three times with 1 ml of RIPA buffer (10mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1 M NaCl, 0.01% NaN_3, 1 mM PMSF, and 1 mM EGTA) and once with 1.0 ml phosphate buffered saline (PBS). Finally the beads were suspended in 1.0 ml of PBS and collected on a 25 mm Fisher Scientific Metrical membrane filter (0.45 mm mesh) with a Hoefer filtration unit. Each tube was washed three times with 1 ml of PBS and the washes passed over the filter. The filter was then solubilized in 5 ml of Ready Protein+ scintillation cocktail and analyzed for radioactivity. Routine control assay mixtures contained no biotinylated peptide and were subtracted out for determining activity. Initially, control assays containing no enzyme were performed which gave high background levels and were thus discontinued.

Recombinant protein as prenyl acceptor. PFT was assayed in some cases using recombinant $p21^{H\text{-}ras}$ as prenyl acceptor (71). Each 25 μl reaction mixture contained, 25-125 μg of cytosolic protein, 50 mM Tris-HCl, pH 7.5, 25 μM $ZnCl_2$, 100 μM $MgCl_2$, 0.7 mM dithiothreitol, 0.5 mM PMSF, 2% glycerol,

10 mM KCl, 11.4 μM $p21^{H\text{-}ras}$ and 0.4 μM [3H]-t,t-farnesyl diphosphate. After 1 h incubation at 37°C, the reaction was stopped by the addition of 0.5 ml SDS (4%, w/v) followed by 0.5 ml ice-cold 30% (w/v) trichloroacetic acid (TCA) with mixing by vortex after each addition. The protein was allowed to precipitate at -20°C for 60-90 min and collected on a 25 mm filter, washed three times with 1.0 ml of 30% TCA and analyzed for radioactivity as described above. Control assay mixtures omitted $p21^{H\text{-}ras}$.

PGGT-I and PGGT-II were measured with recombinant maltose binding protein (MBP) chimeric proteins, MBP-G25K, MBP-rab 1B, rab 5, and rab 6 according to Kinsella and Maltese (69). A 50 μl reaction mixture contained 200 μg of cytosolic protein, 25 mM Tris-HCl, pH 7.5, 25 μM $ZnCl_2$, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μM leupeptin, 0.1 μM pepstatin, 50-170 pmol of recombinant protein, and 2.0 μM [^{3}H]-t,t,t-geranylgeranyl diphosphate. After 1 h at 37°C, the reaction was stopped by the addition of 500 μl ice cold acetone and subjected to centrifugation at top speed for 15 min in a microfuge. The pellet was dissolved in Laemmli sample buffer and subjected to SDS-PAGE (102). Slices (3 mm) for each lane were incubated with 250 μl of H_2O_2 at 60°C for 18 h, mixed with 10 ml scintillation cocktail (Scintiverse II, 33% triton X-100) and analyzed for radioactivity.

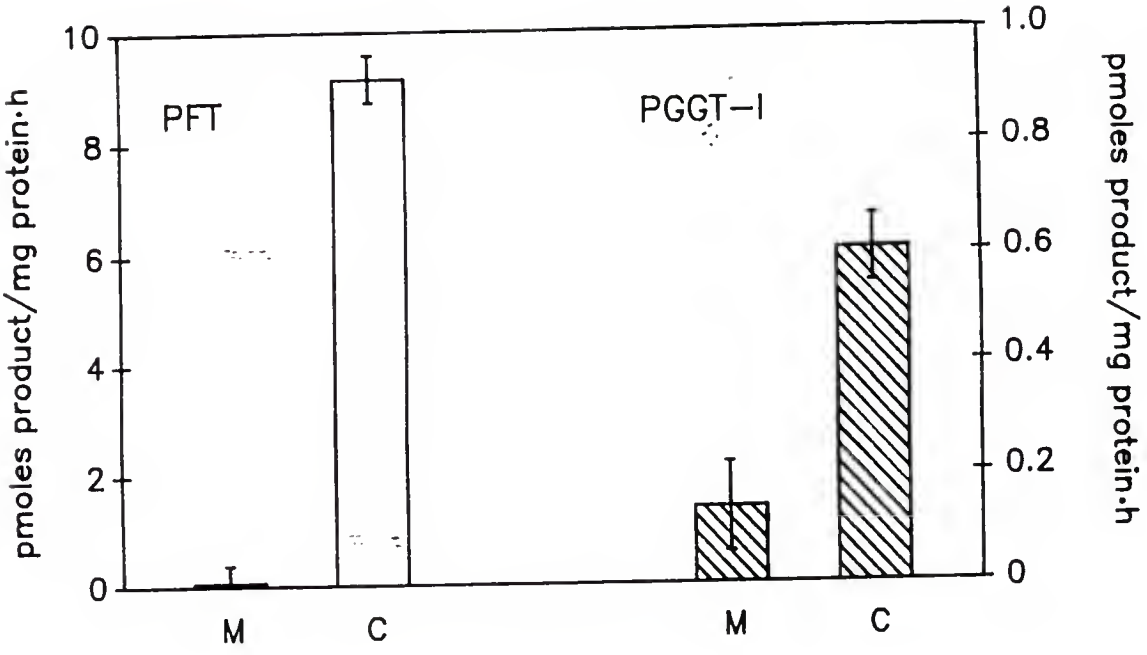
Results

Testicular protein prenyl transferases. Others have shown that both enzymatic activities have an absolute requirement for divalent metal ions (71, 94-96). A limited number of experiments, not detailed here, showed divalent cation requirements for PFT and PGGT-I similar to those described in the literature.

PFT and PGGT-I activity was analyzed in subcellular fractions of rat testicular cells. The cells were homogenized in a hypotonic buffer containing 10% glycerin and then subjected to centrifugation as described in the methods to yield cytosolic and microsomal fractions. The activities of both PFT and PGGT-I from decapsulated testes are exclusively located in the soluble fraction (Fig. 2-2). The specific activity of PGGT-I measured with the biotinylated peptide Bt-KKFFCAIL was repeatedly 15-40 fold less than that of PFT measured with Bt-KTKCVIS. The dependence of farnesylation on the concentration of testicular cytosolic protein was measured for both recombinant $p21^{ras}$ and the biotinylated peptide, Bt-KTKCVIS, using 0.4 μM [^{3}H]-farnesyl diphosphate (Fig. 2-1A,B). Similarly, the dependence of geranylgeranylation on cytosolic protein was determined with Bt-KKFFCAIL using 2.0 μM [^{3}H]-geranylgeranyl diphosphate (Fig. 2-1D). Most of the subsequent assays utilized 125 μg and 25 μg of cytosolic testicular protein for PFT assays with $p21^{ras}$ and Bt-KTKCVIS, respectively; and 50 μg of protein for PGGT-I assays with

Figure 2-2. Cytosolic Localization for PFT and PGGT-I Activities.

Cytosolic (C) and membrane (M) protein were fractionated from decapsulated testes of 40 day old rats as described in the methods. The incubations for PFT assays contained 30 μM Bt-KTKCVIS, 0.4 μM [^{3}H]-FPP, and 25 μg of protein; incubations for PGGT-I assays contained 30 μM Bt-KKFFCAIL, 2.0 μM [^{3}H]-GGPP, and 50 μg of protein. PFT assays were performed in duplicate. PGGT-I values represent triplicate assays.

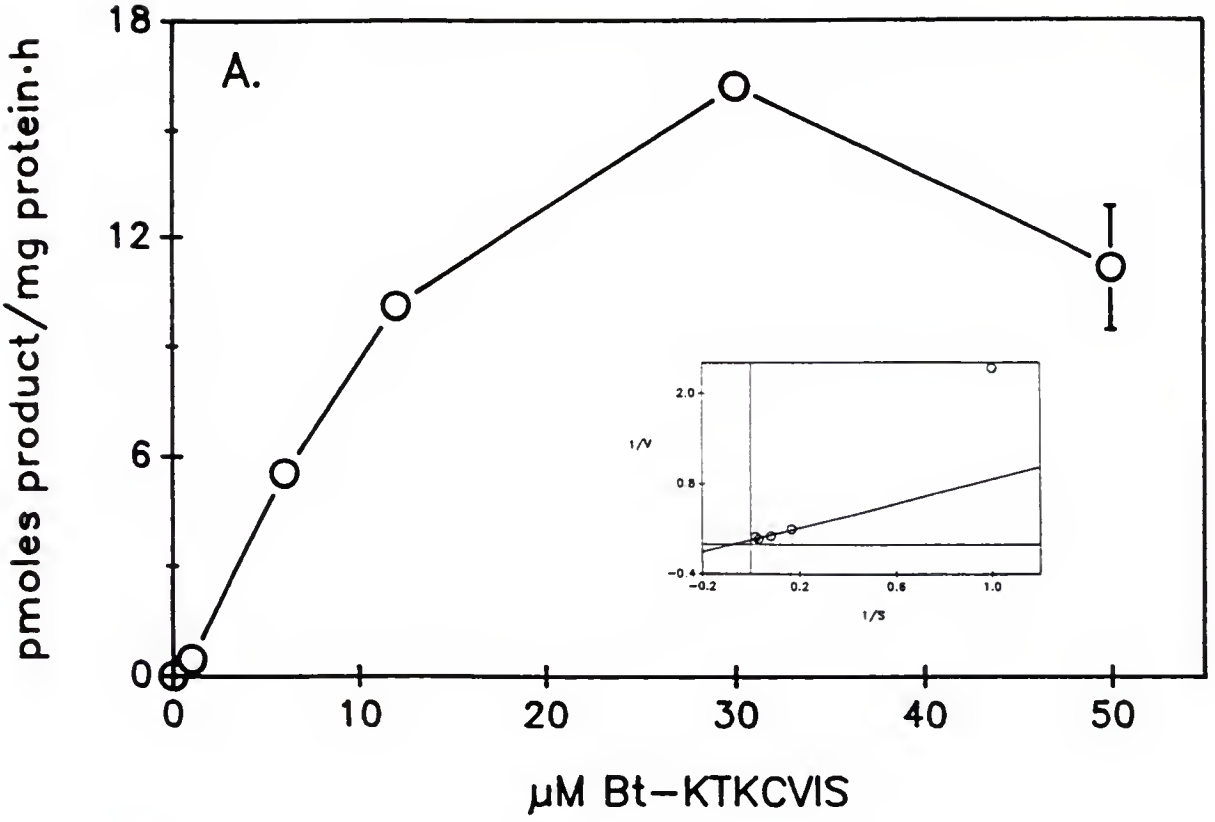

pmoles product/mg protein·h
10
8
6
4
2
0
PFT
PGGT–I
1.0
0.8
0.6
0.4
0.2
0
pmoles product/mg protein·h
M
C
M
C

Bt-KKFFCAIL. The specific activity of PGGT-I was about 40-fold lower than that of PFT in adult rat testicular cells. Linearity of PFT activity with cytosolic protein concentration was also established for preparations of seminiferous epithelium and spermatogenic cells from 23 day old rats (Fig. 2-1C). The assay was linear up to 25 μg of protein for both cell populations and furthermore; both these cell populations appeared to have the same levels of PFT activity.

Optimal substrate concentrations for the biotinylated peptides and the tritiated allylic substrates were examined. PFT activity showed optimal activity at 30 μM Bt-KTKCVIS (Fig. 2-3A). A double reciprocal plot gave an estimated K_m of 14 μM (Fig. 2-3A,insert). PFT assays with varying concentrations of [^{3}H]-FPP showed that 0.4 μM FPP was sufficient to saturate the enzyme (Fig. 2-3B). The K_m for FPP was established to be 0.2 μM (Fig. 2-3B,insert). Optimal PGGT-I activity was achieved at 30 μM Bt-KKFFCAIL and 2.0 μM with GGPP (Fig. 2-4A,B). Because of the lower activity with PGGT-I and increased scatter in the data, only K_m estimates of 18 μM and 1.7 μM can be made for the peptide and GGPP, respectively. A comparison of these K_ms with those reported for similar enzymes are depicted in Table 2-1. All assays were performed at saturating concentrations of biotinylated peptides and allylic substrates and within the linear response range for cytosolic protein, except for the

Figure 2-3. Peptide and Allylic Substrate Concentration Dependence for PFT Activity.

PFT incubations contained 25 μg of cytosolic protein from decapsulated testes of a 26 day old and were assayed as described in the methods with various peptide or allylic concentrations. The concentration of Bt-KTKCVIS (Panel A) or [^{3}H]-FPP (Panel B) was varied as indicated. Insets in Panel A and Panel B show double reciprocal plots for the PFT substrates. All points represent the average of duplicate assays. Error bars not seen lie within the symbol.


A.
pmoles product/mg protein·h
18
12
6
0
0
10
20
30
40
50
μM Bt–KTKCVIS
1/V
2.0
0.8
-0.4
-0.2
0.2
0.6
1.0
1/S

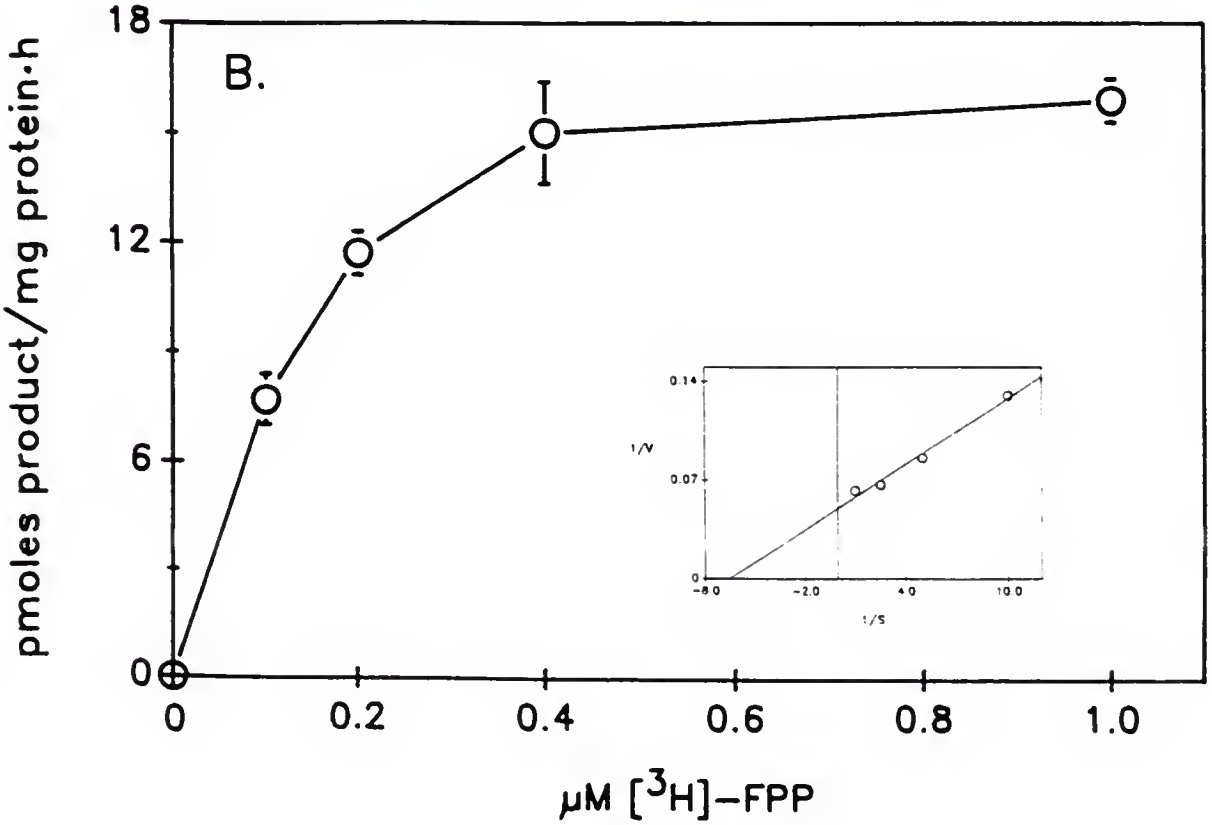
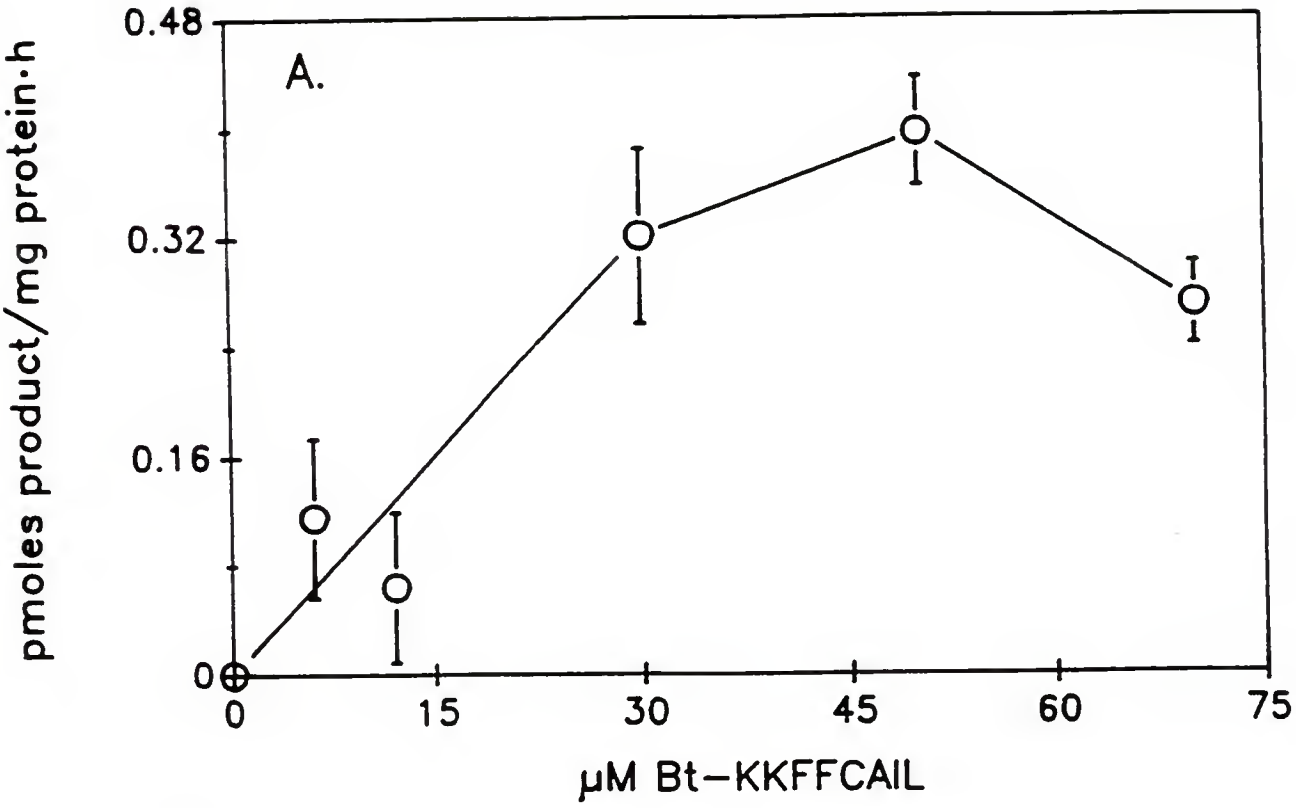

B.
pmoles product/mg protein·h
18
12
6
0
0
0.2
0.4
0.6
0.8
1.0
μM [3H]–FPP
1/V
0.14
0.07
0
-8.0
-2.0
4.0
10.0
1/S

Figure 2-4. Peptide and Allylic Substrate Concentration Dependence for PGGT-I Activity.

PGGT-I incubations contained 50 μg of cytosolic protein from decapsulated testes of a 26 day old rat and were assayed as described in the methods with various concentrations of peptide or allylic substrates. The concentration of Bt-KKFFCAIL (Panel A) or [^{3}H]-GGPP (Panel B) was varied as indicated. All data points represent the average of triplicate assays.


A.
0.48
0.32
0.16
0
pmoles product/mg protein·h
0
15
30
45
60
75
μM Bt–KKFFCAIL

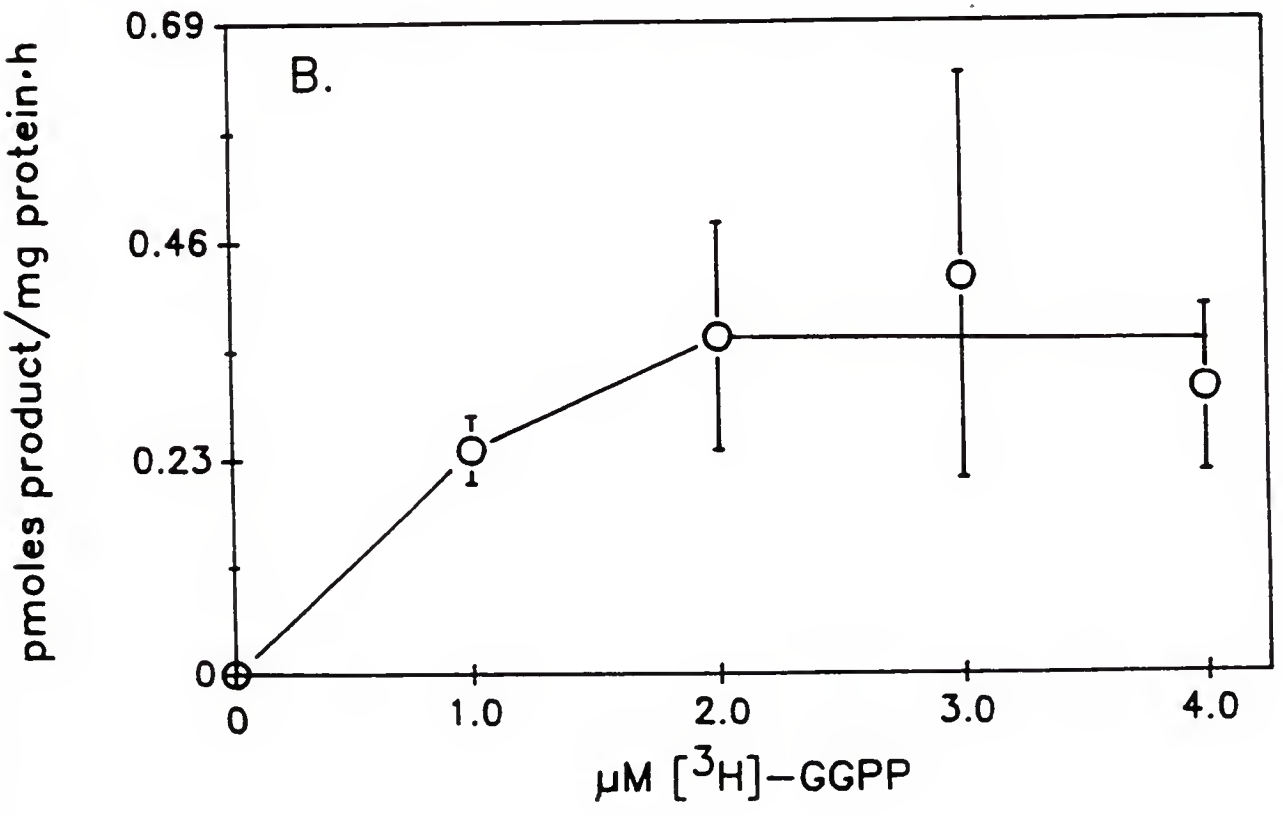

B.
0.69
0.46
0.23
0
pmoles product/mg protein·h
0
1.0
2.0
3.0
4.0
μM [3H]–GGPP

Table 2-1

Approximate K_ms (μM) of Protein, Peptide and Allylic Substrates for PFT, PGGT-I and PGGT-II[a]

PFT	Protein or Peptide	FPP	Source	Ref[b]
Bt-KTKCVIS	14	0.2	cytosol, rat testes	this study
H-Ras-CVLS	2.4	2	cytosol, TT cells	94
H-Ras-CVLS	5	0.5	pp[c], rat brain	71
H-Ras-CVLS	3.5	0.25	pp, bovine brain	72
Bt-NRSCAIS	3		pp, bovine brain	77
H-Ras-CVLS	20	0.4	pp, bovine brain	78
H-Ras-CVLS	8		pp, bovine brain	80
H-Ras-CVLS	0.13-0.63	0.04	p[d], bovine brain	107
H-Ras-CVLS		<0.1	p, rat brain	75
PGGT-I	Protein or Peptide	GGPP	Source	Ref.
Bt-KKFFCAIL	18	1.7	cytosol, rat testes	this study
Ras-CAIL	0.5	0.09	pp, bovine brain	72
Bt-KKFFCAIL	5		pp, bovine brain	77
RhoA-CLVL	1	3	pp, bovine brain	78
Ras-CVLL	1.5	0.3	pp, bovine brain	80
PGGT-II	Protein	GGPP	Source	Ref.
YPT1	0.3	0.07	pp, bovine brain	72
Rab3A-CAC	3	2	pp, bovine brain	143
Rab3A-CAC	0.2	0.2	p, rat brain	86

[a]Incubation conditions for PFT and PGGT-I referenced to this study were the same as described in the methods.
[b]Ref refers to the reference where the K_m information was obtained.
[c]pp = partially purified
[d]p = purified to homogeneity

experiment where recombinant protein was used as the prenyl acceptor.

Time dependence for PFT and PGGT-I activities was measured with the biotinylated peptide substrates. PFT activity was linear for at least 60 min (Fig. 2-5). Sixty min was also an appropriate incubation time for PGGT-I activity (data not shown).

Evaluation of the ability of related but different peptides to act as substrates or inhibitors of enzyme activity is useful in establishing enzyme specificity. Since PFT and PGGT-I were assayed from the same cytosolic preparation it was necessary to demonstrate separate activities. Farnesyl transferase activity showed a strong specificity for the peptide substrate Bt-KTKCVIS, whereas Bt-KKFFCAIL, the PGGT-I substrate, was not farnesylated (Fig. 2-6). In contrast, for assessing geranylgeranyl transferase activity, Bt-KKFFCAIL was the best substrate but Bt-KTKCVIS was also geranylgeranylated to the extent of 50% of Bt-KKFFCAIL. Although cross-reactivity of substrates was observed, it was concluded that distinct activities of PFT and PGGT-I are being measured when Bt-KTKCVIS and Bt-KKFFCAIL, respectively are used as substrates. This conclusion is discussed in more detail in the discussion section of the Chapter.

Figure 2-7A shows inhibition of PFT by increasing concentrations of the acetylated peptide, Ac-KTKCVIS. Only

Figure 2-5. Time Dependence for PFT Activities.

PFT activity was assayed as described in the methods with 30 μM Bt-KTKCVIS, 0.4 μM [^{3}H]-FPP and 125 μg of decapsulated testicular cytosolic protein from a 58 day old rat at the time points indicated. Each time point was performed in duplicate.

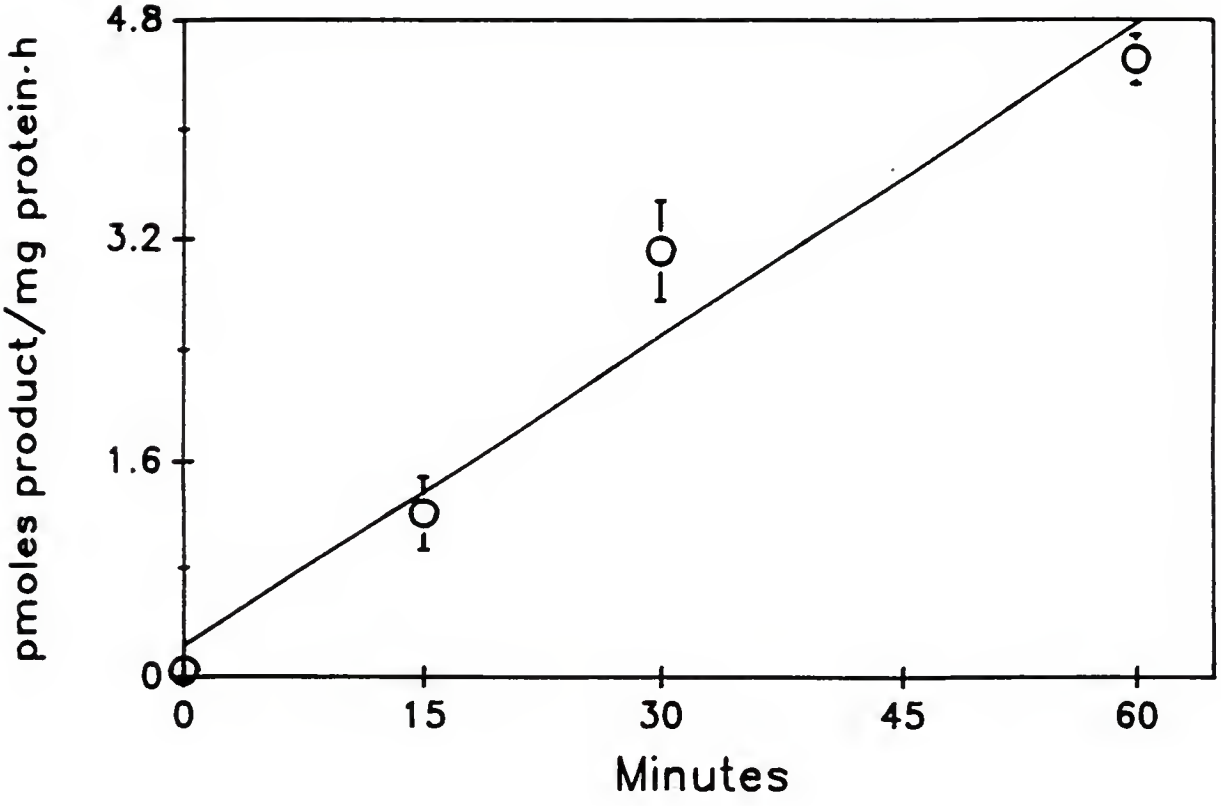

4.8
3.2
1.6
0
pmoles product/mg protein·h
0
15
30
45
60
Minutes

Figure 2-6. Peptide Specificity of PFT and PGGT-I.

PFT and PGGT-I activities in decapsulated testes from 17 day old rats were each assessed using 30 μM Bt-KTKCVIS or 30 μM Bt-KKFFCAIL with [^{3}H]-FPP (and 25 μg cytosolic protein) or [^{3}H]-GGPP (and 50 μg cytosolic protein) as described in the methods. Controls for PFT and PGGT-I assays in which Bt-peptide were omitted gave values of 1.7 pmoles/mg and 0.5 pmoles/mg, respectively which have been subtracted. PFT assays were performed in duplicate and PGGT-I assays were performed in triplicate.

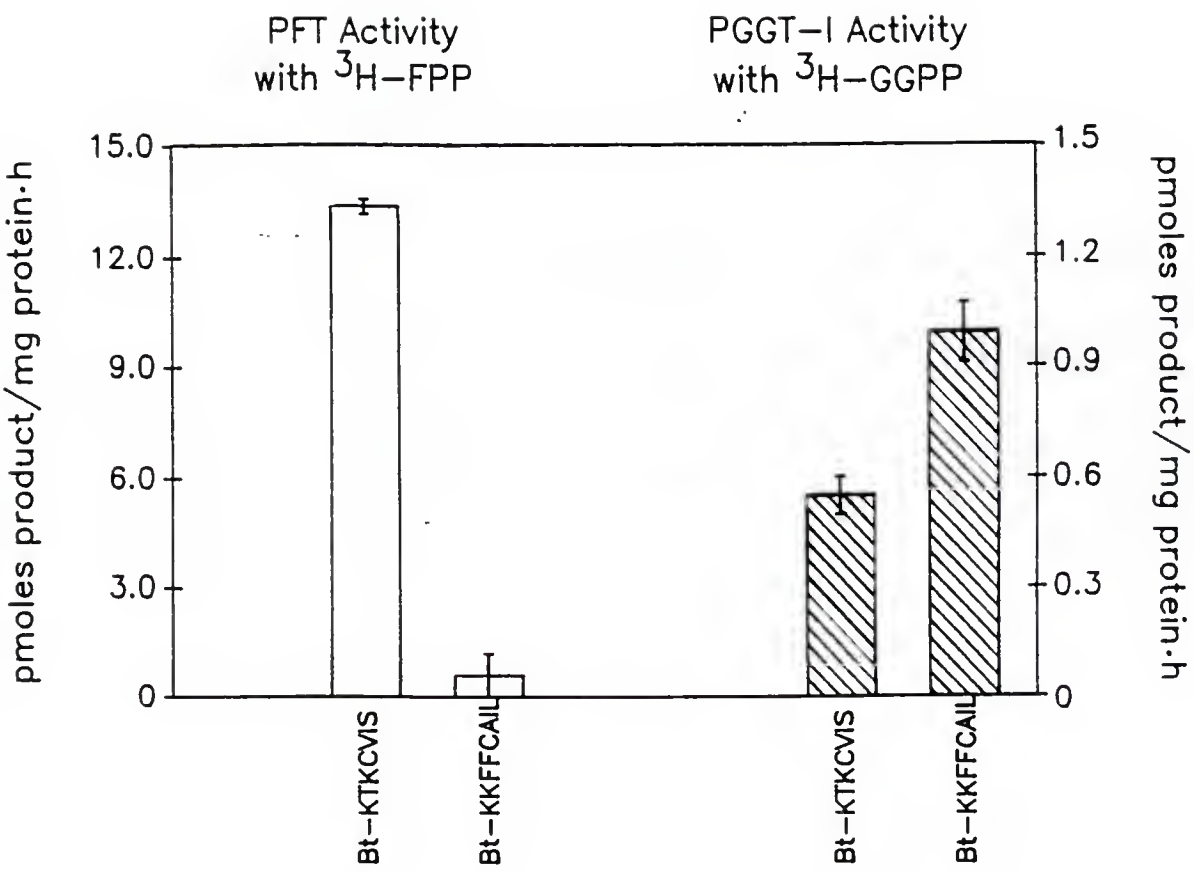
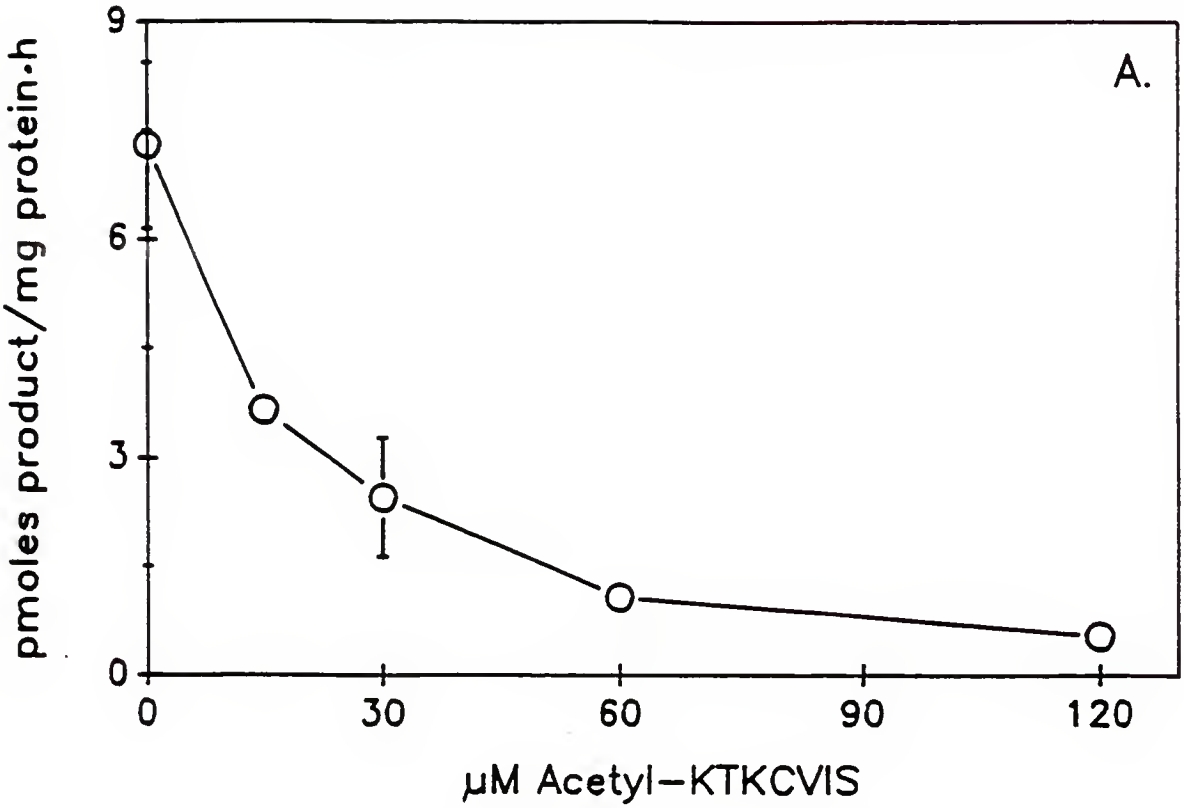
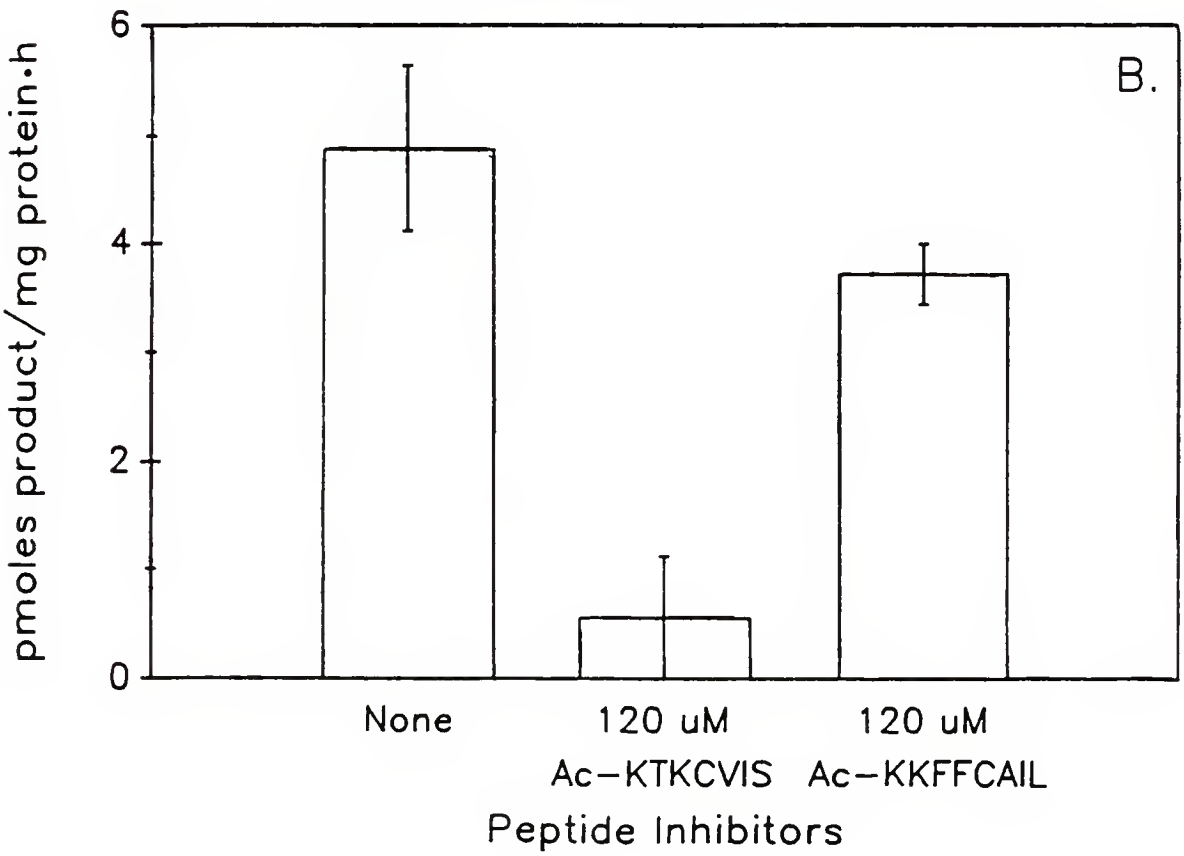

PFT Activity
with ^{3}H–FPP
PGGT–I Activity
with ^{3}H–GGPP
pmoles product/mg protein·h
15.0
12.0
9.0
6.0
3.0
0
1.5
1.2
0.9
0.6
0.3
0
Bt–KTKCVIS
Bt–KKFFCAIL
Bt–KTKCVIS
Bt–KKFFCAIL

Figure 2-7. Inhibition of Farnesylation of Bt-KTKCVIS with N-acetyl-KTKCVIS.

PFT activity was assayed with 25 μg of decapsulated testicular cytosolic protein from a 45 day old rat as described in the methods with (A) 30 μM Bt-KTKCVIS and varying concentrations of N-acetyl-KTKCVIS or (B) 30 μM BT-KTKCVIS and either 120 μM N-acetyl-KTKCVIS or 120 μM N-acetyl-KKFFCAIL. All assays were performed in duplicate, error bars not seen lie within the symbol.


A.
pmoles product/mg protein·h
9
6
3
0
0
30
60
90
120
μM Acetyl–KTKCVIS

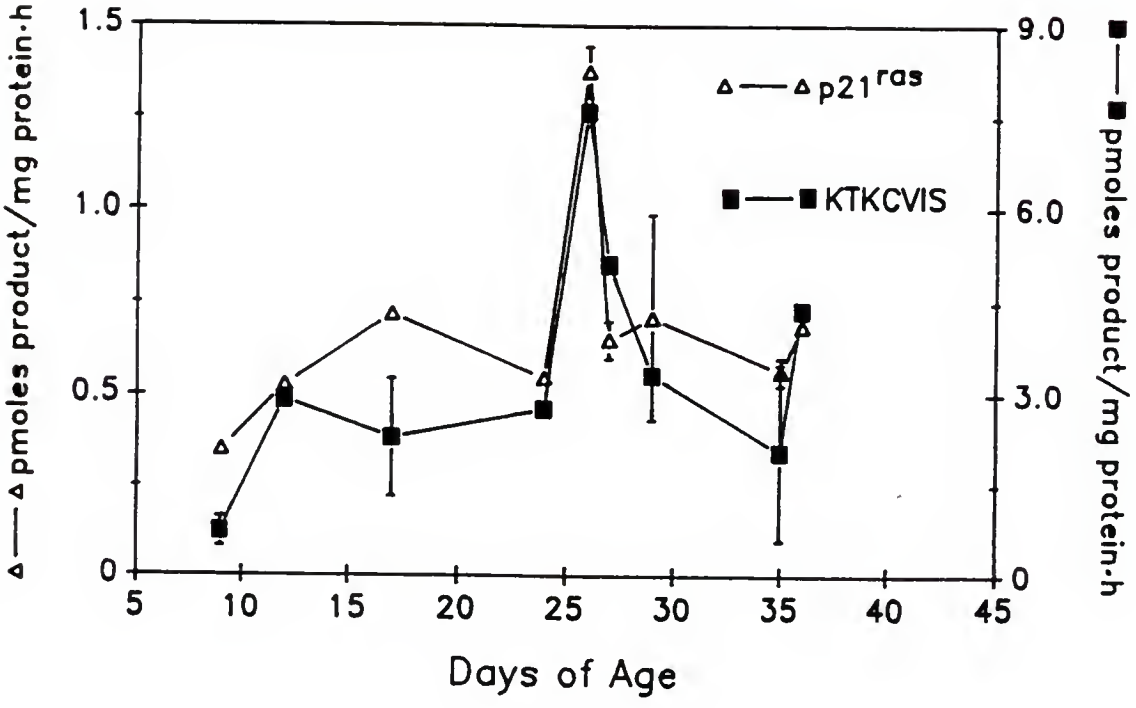

B.
pmoles product/mg protein·h
6
4
2
0
None
120 uM Ac–KTKCVIS
120 uM Ac–KKFFCAIL
Peptide Inhibitors

15 μM was necessary to inhibit the prenylation of Bt-KTKCVIS by half, which suggests the acetylated peptide may be a better substrate for PFT than the biotinylated substrate. Similar K_ms indicate that the biotinyl group has little effect on the suitability of Bt-KTKCVIS as a substrate. The specificity of inhibition of PFT by peptides is further demonstrated in Fig. 2-7B, since the same concentration of Ac-KKFFCAIL failed to appreciably inhibit the prenylation of Bt-KTKCVIS under conditions where Ac-KTKCVIS gave nearly complete inhibition.

Age-dependent activity of protein prenyl transferases. In order to determine if protein prenyl transferase activities change during distinct stages of spermatogenesis, prepuberal animals were utilized. This is the only period when specific cell types emerge for the first time during differentiation. Cytosolic fractions of the decapsulated testes of different aged rats were assayed for PFT. PFT specific activity measured with either recombinant $p21^{H\text{-}ras}$ or Bt-KTKCVIS as polypeptide substrate showed a peak in activity at 26 days of age when measured with 125 μg of protein (Fig. 2-8). This indicates the biotinylated peptide is a representative substrate for PFT and can be used to accurately measure changes in the enzyme capacity to farnesylate. Activity at the peak was 2-3 fold higher than that observed at earlier or later ages. The specific

Figure 2-8. Prepuberal Age Study of PFT Activity Comparing Bt-KTKCVIS Peptide with $p21^{H\text{-}ras}$ Protein as Substrates.

Activity was assayed using 125 μg of decapsulated testicular cytosolic protein taken from different aged prepuberal rats and either 30 μM Bt-KTKCVIS or 11.4 μM $p21^{H\text{-}ras}$ as described in the methods. All values for data points measured with $p21^{H\text{-}ras}$ were performed in duplicate; error bars not seen lie within the symbol. Data points measured with Bt-KTKCVIS were averaged from 1-3 experiments, shown in Fig. 2-9, each performed in duplicate.


Δ—Δ pmoles product/mg protein·h
■—■ pmoles product/mg protein·h
Δ—Δ p21ras
■—■ KTKCVIS
1.5
1.0
0.5
0
9.0
6.0
3.0
0
5
10
15
20
25
30
35
40
45
Days of Age

activities of PFT in the cytosolic fraction from animals aged 45-105 days was not greater than that demonstrated at 17 days of age (Fig. 2-9). It was of interest to determine if the peak of activity in 26 day old animals was contributed to by the changing population of germ cells. Germ cells were isolated from Sertoli cells and interstitial cells and tested for prenyl transferase activity. An age dependent peak of PFT activity at 23 days of age was observed with isolated spermatogenic cells (Fig. 2-10A). PGGT-I specific activity showed a peak that was coincident with age to that found for PFT in spermatogenic cells (Fig. 2-10B). Although the specific activity of PGGT-I was twenty-five fold less than that for PFT, the peak in activity at 23 days of age was still 2-3 fold higher than that seen in animals several days younger or older.

The other known enzyme that geranylgeranylates proteins, PGGT-II, was assayed in 17, 23, and 60 day old animals using chimeras of recombinant maltose binding protein (MBP) and rab 1B, rab 5, and rab 6, which have the carboxy terminal sequences indicated in Table 2-2. Recombinant proteins specifically prenylated by either PGGT-I or PFT were utilized as controls. These proteins were a MBP-G25K chimera with a carboxy terminal sequence CVLL and $p21^{ras}$ (not a chimera) with the carboxy terminal sequence CVIS. $p21^{ras}$ was clearly labelled with [^{3}H]-FPP by cytosol from 60 day old animals. MBP-G25K was labelled with [^{3}H]-

Figure 2-9. Prepuberal Changes in PFT Activity in Comparison with Adult Activity.

PFT activity, assayed from decapsulated testes of adult rats using 125 μg of cytosolic protein and 30 μM Bt-KTKCVIS, was plotted with data from Fig. 2-8. Numbers in paranthesis represent the number of assays performed at each age and the error bars demonstrate variances between experiments. Each assay was performed in duplicate.

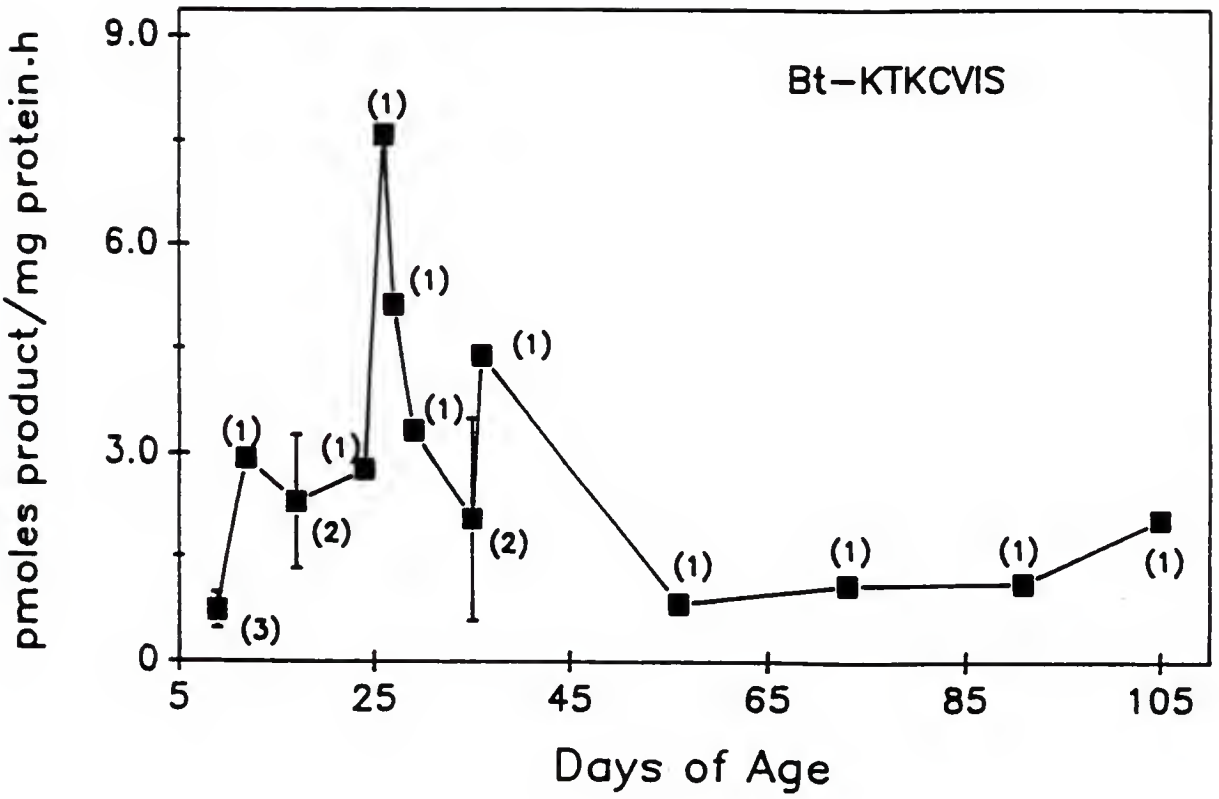

Bt–KTKCVIS
pmoles product/mg protein·h
9.0
6.0
3.0
0
5
25
45
65
85
105
Days of Age
(1)
(1)
(1)
(1)
(1)
(1)
(2)
(2)
(3)
(1)
(1)
(1)
(1)

Figure 2-10. Age Dependent Changes in Protein PFT and PGGT-I Activities in Spermatogenic Cells.

Spermatogenic cells were isolated as described in the methods. Activity was assayed using 60-80 μg of cytosolic protein and either 30 μM Bt-KTKCVIS (PFT, Panel A) or 30 μM Bt-KKFFCAIL (PGGT-I, Panel B). PFT assays were performed in duplicate; error bars not seen lie within the symbol. PGGT-I assays were performed in triplicate.

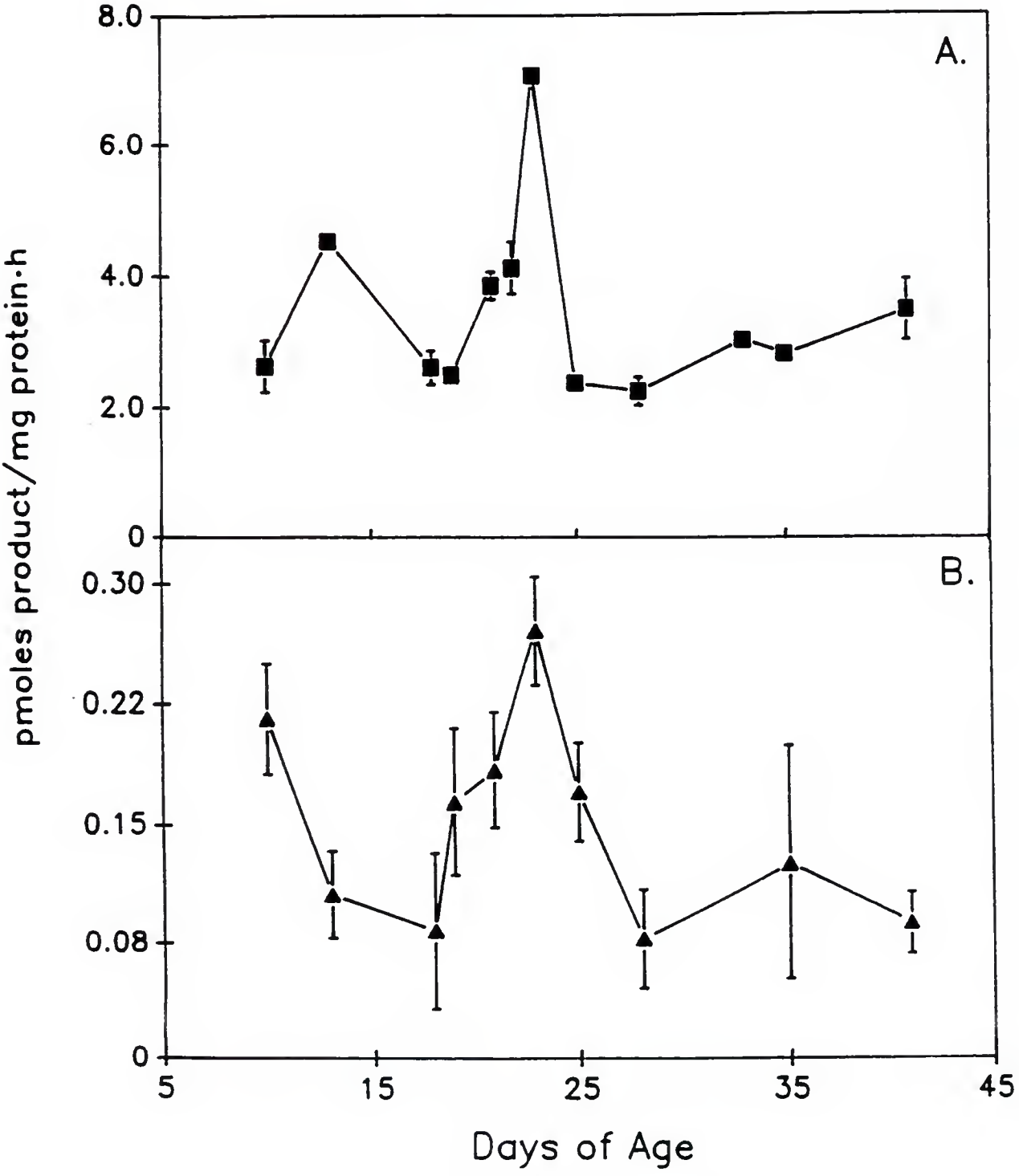

8.0
6.0
4.0
2.0
0
A.
0.30
0.22
0.15
0.08
0
B.
pmoles product/mg protein·h
5
15
25
35
45
Days of Age

Table 2-2

Age Study of Prenylation of Recombinant Proteins[a]

Recombinant protein	17 day old (pmoles/mg)	23 day old (pmoles/mg)	60 day old (pmoles/mg)
$p21^{ras}$ (-CVIS)	0.320[b]	1.322[b]	0.554[b]
			0.701
MBP-G25K (-CVLL)	0.345	0.602	0.448
MBP-rab1B (-GGCC)	0.078	0.070	0.034
MBP-rab5 (-CCSN)	0.070	0.072	0.047
MBP-rab6 (-GCSC)	0.092	0.073	0.032

[a]Incubation conditions for prenylation assays were performed as described in the methods for recombinant proteins.
[b]These data were obtained from PFT assays using TCA precipitation and filter binding. All others were obtained from radioactivity analysis of dissolved PAGE slices.

GGPP by cytosol from 23 day old animals to a level 1.5- to 2-fold higher than that seen with cytosol from 17 and 60 day old animals. This substantiates the peak observed for PGGT-I activity in 23 day old animals measured with the biotinylated peptide Bt-KKFFCAIL. In contrast, activity for the PGGT-II enzyme, measured with MBP-rab proteins, were very low with no age dependent differences. Since the recombinant proteins were present in E. coli. extracts, accurate measurements of the protein concentrations were not possible. Adequate levels of protein substrate were believed to be present since no effect on the levels of activity were seen on doubling the amount of E. coli extracts.

The results of these studies were attained at relatively high concentrations of cytosolic protein (60-125 μg) which is near the limit of the linear response in some cases. This could have affected the position and extent of the peak of activity. However, the peak of PFT activity in decapsulated testes at 23 days of age was confirmed by testing at lower protein concentrations (25 μg). A two-fold increase in cytosolic activity was observed going from 17 days of age (3.4 ± 0.6 pmoles/mg protein x h) to 23 days days of age (6.3 ± 0.2 pmoles/mg protein x h) with Bt-KTKCVIS as substrate. A similar decline was seen going from 23 to 26 days of age (4.2 ± 0.4 pmoles/mg protein x h).

PFT activity differences were also assessed between decapsulated testes and germ cells at the lower protein

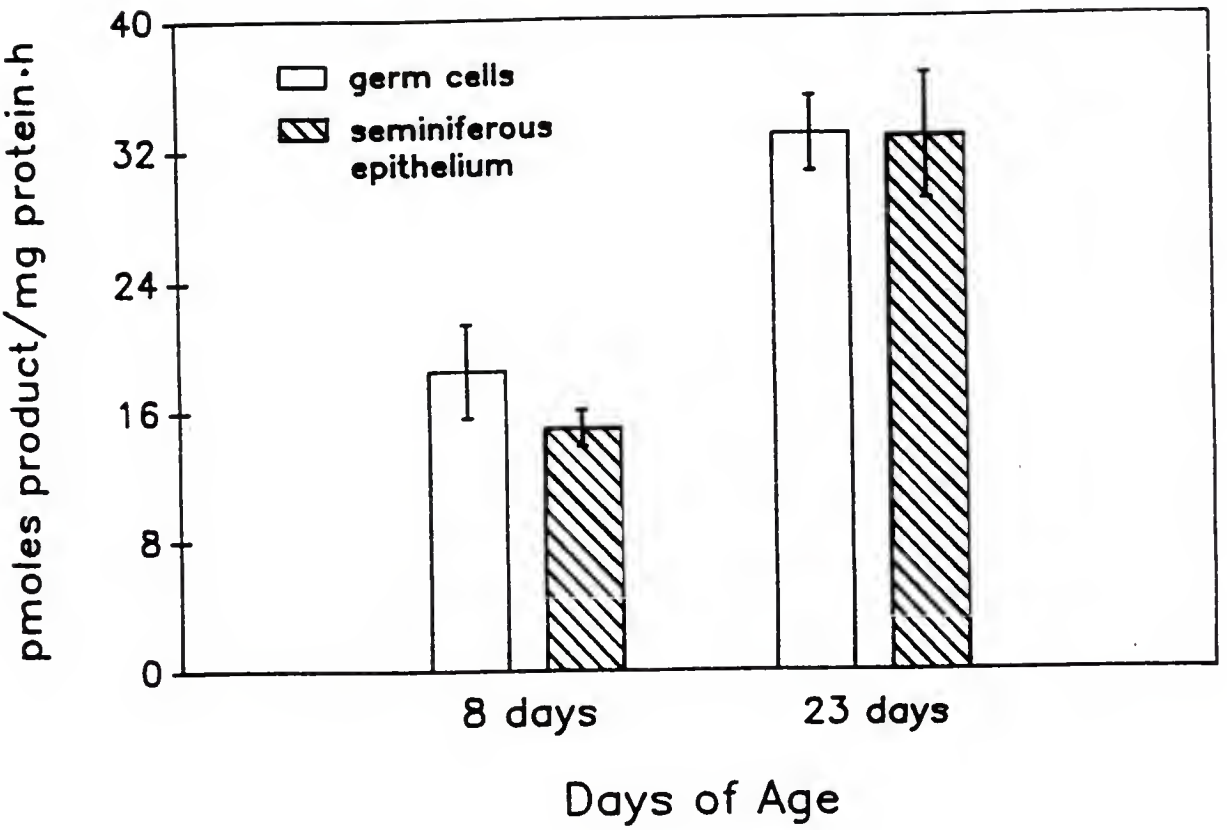
concentration (25 μg). The specific activity of cytosolic PFT in decapsulated testes from 23 day old rats was 6.3 ± 0.2 pmoles/ mg protein x h, whereas the activity of spermatogenic cells was 2-fold higher with 13.7 ± 0.9 pmoles product/mg protein x h. At other ages tested, PFT activity in spermatogenic cells was also higher than in decapsulated testes.

The contribution of the somatic Sertoli cell to the age-dependent changes in PFT activity were assessed by assaying PFT in germ cells and seminiferous epithelium at 8 and 23 days of age (Fig. 2-11). Similar to previous results, a 2-fold increase in activity occurred from 8 to 23 days of age in germ cells and seminiferous epithelium as shown in Fig. 2-1C. PFT specific activities between germ cells and seminiferous epithelium from 23 day old animals were equal. Furthermore, since Fig. 2-11 shows that both cell populations increase in PFT activity with little difference in specific activity between them at each age tested, this suggests that the Sertoli cells, which are the somatic cells of the seminiferous epithelium, have a similar specific activity and that they cycle in farnesylation capacity coincidently with the germ cells. Thus, somatic cells make an equal contribution to activity.

Spermatogenic cell type differences in protein prenyl transferase activities. In order to assess the spermatogenic

Figure 2-11. PFT Activity in Spermatogenic Cells and Seminiferous Epithelium.

Seminiferous epithelium and spermatogenic cells were isolated from the same group of rats as described in the methods. PFT activity was assayed with 25 μg of cytosolic protein and 30 μM Bt-KTKCVIS. Bar values represent the average of duplicate assays.

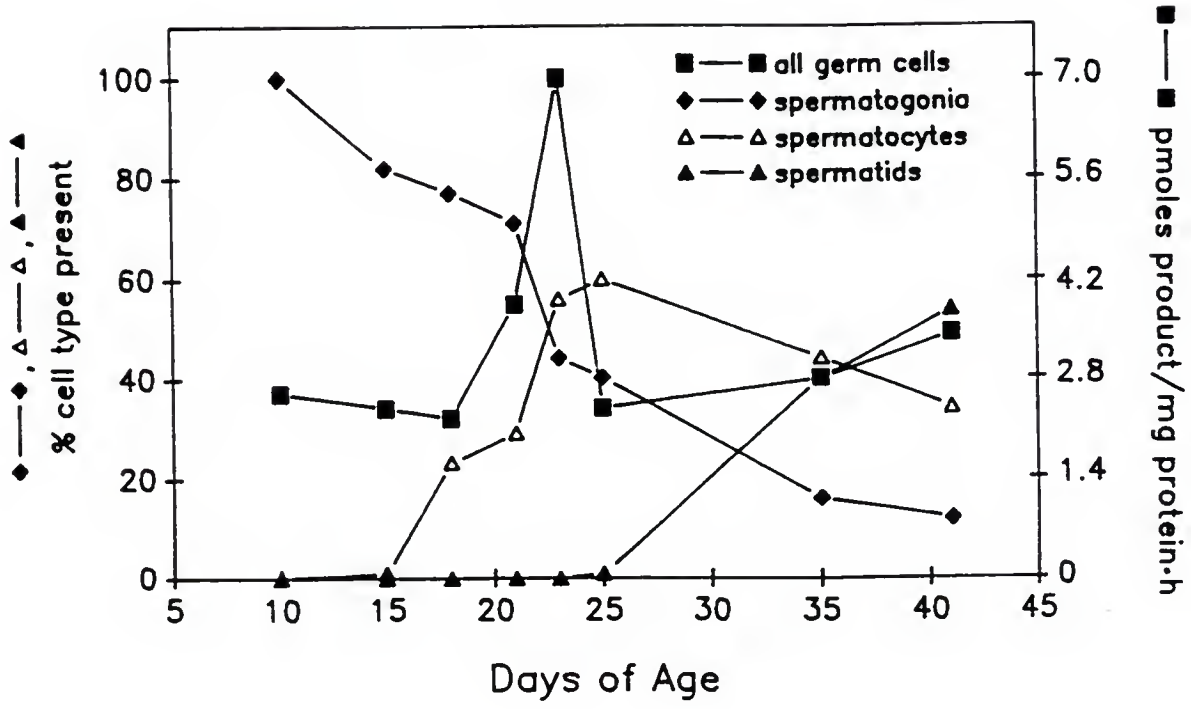

40
32
24
16
8
0
pmoles product/mg protein·h
germ cells
seminiferous epithelium
8 days
23 days
Days of Age

cell types present at various days of age in the prepuberal rat, data from Zhengwei et al. (103) on quantitation of cell types in the developing rat testes were converted to give the percentage of each spermatogenic cell type present relative to the whole population of spermatogenic cells at particular days of age. These percentages were plotted (Fig. 2-12) along with PFT specific activities from rats of the same days of age (data from Fig. 2-10A). At 9 days of age the spermatogonia are the only spermatogenic cell type present, so they represent 100% of the germ cells. As the primary spermatocytes appear at day 15 and rise in numbers, the spermatogonia decrease relative to the spermatocytes. The primary spermatocytes peak at 23-25 days as the most prevalent cell types present. The round spermatids appear at day 25 and quickly accumulate in numbers. By day 40, just before puberty, the spermatids are the most highly represented of the spermatogenic cells. The round spermatids continue to increase in numbers well past puberty in the adult rat. These data show a correlation of the rise in PFT activity with the rise in numbers of spermatocytes.

In order to directly assess the PFT activity in selected types of germ cells, it was necessary to physically separate them. The Sta Put unit gravity cell separator allowed the separation and identification of the pachytene spermatocytes and the round spermatids. Spermatozoa, mature sperm, were isolated from the epididymis of the adult rat.

Figure 2-12. Age Dependent Changes of Spermatogenic Cell Population and PFT Activity.

PFT activity in prepuberal spermatogenic cells from Fig. 2-10, Panel A, is plotted along with the percentage of indicated cell types present in the spermatogenic cell population from Zhengwei et al., 1990 (103).


all germ cells
spermatogonia
spermatocytes
spermatids
% cell type present
pmoles product/mg protein·h
Days of Age
100
80
60
40
20
0
7.0
5.6
4.2
2.8
1.4
0
5
10
15
20
25
30
35
40
45

Measurements of specific activity of PFT were similar between adult pachytene spermatocytes and round spermatids (Fig. 2-13, P and R), whereas the specific activity in spermatozoa was negligible (Fig. 2-13, SZ). Pachytene spermatocytes were also isolated from 23 day old animals and analyzed for PFT activity. The prepuberal spermatocytes showed a 2 fold higher level of PFT than the adult spermatocytes, yet the whole spermatogenic cell mix from the same 23 day old animals revealed four fold higher PFT specific activity (Fig. 2-13, 23P and 23W). Thus, the pachytene spermatocytes alone were not responsible for the increase in PFT specific activity at 23 days. The specific activities of PGGT-I is not significantly different among any of these cell types (Fig. 2-14). In order to further identify the cell type responsible for the peak of PFT activity in 23 day old spermatogenic cells, pachytene spermatocytes and all Sta Put fractions eluted before and after the pachytene spermatocytes were collected, pooled, and assayed for PFT activity. Figure 2-15 shows that the cells with the highest activity appear in the pooled fraction eluted just after the pachytene spermatocytes. This pooled fraction represents a mixture of cell types all of which are slightly lighter in density and smaller in size (as seen by light microscopy) than the pachytene spermatocytes.

Figure 2-13. PFT Activity in Isolated Pachytene Spermatocytes and Round Spermatids in Adult and 23 Day Old Rats.

Pachytene spermatocytes (P), round spermatids (R) and spermatozoa (SZ) were isolated from 78 day old rats, as described in the methods. Pachytene spermatocytes (23P) were isolated from spermatogenic cells of 23 day old rats (23W) by the same proceedure. PFT activity was assayed using 25 μg of cytosolic protein and 30 μM Bt-KTKCVIS. The data represent duplicate assays in one experiment, which is representative of 3 separate experiments.

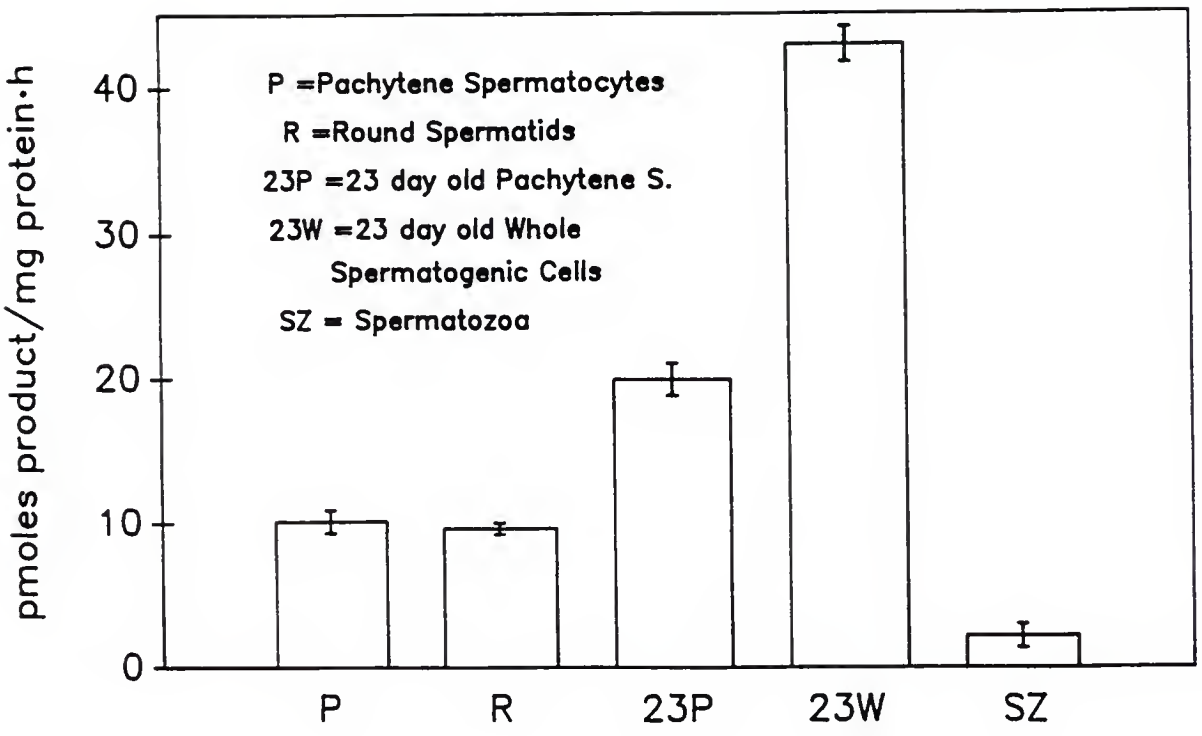

pmoles product/mg protein·h
40
30
20
10
0
P =Pachytene Spermatocytes
R =Round Spermatids
23P =23 day old Pachytene S.
23W =23 day old Whole
Spermatogenic Cells
SZ = Spermatozoa
P
R
23P
23W
SZ

Figure 2-14. PGGT-I Activity in Isolated Pachytene Spermatocytes and Round Spermatids in Adult and 23 Day Old Rats.

Spermatogenic cell types were isolated from 78 and 23 day old rats, as described in the legend of Fig. 2-13. PGGT-I activity was assayed using 50 μg of cytosolic protein and 30 μM Bt-KKFFCAIL. The data represent triplicate assays in one experiment, which is representative of 3 separate experiments.

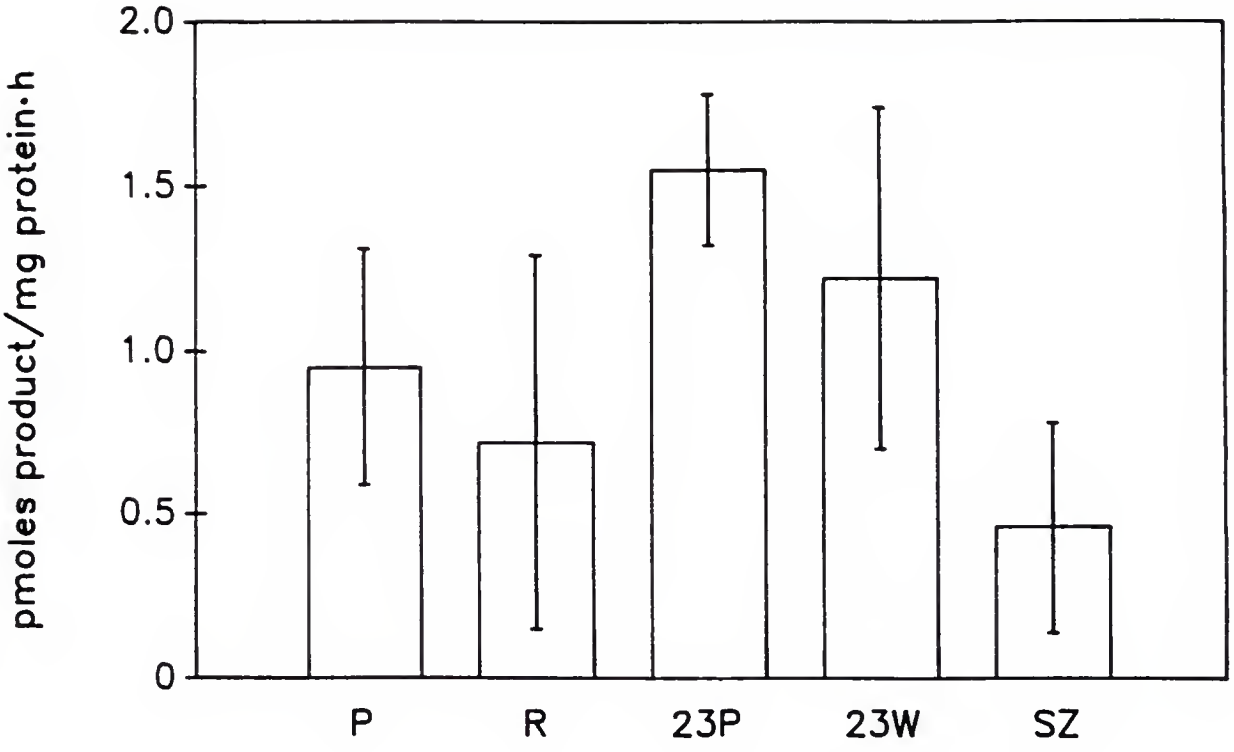
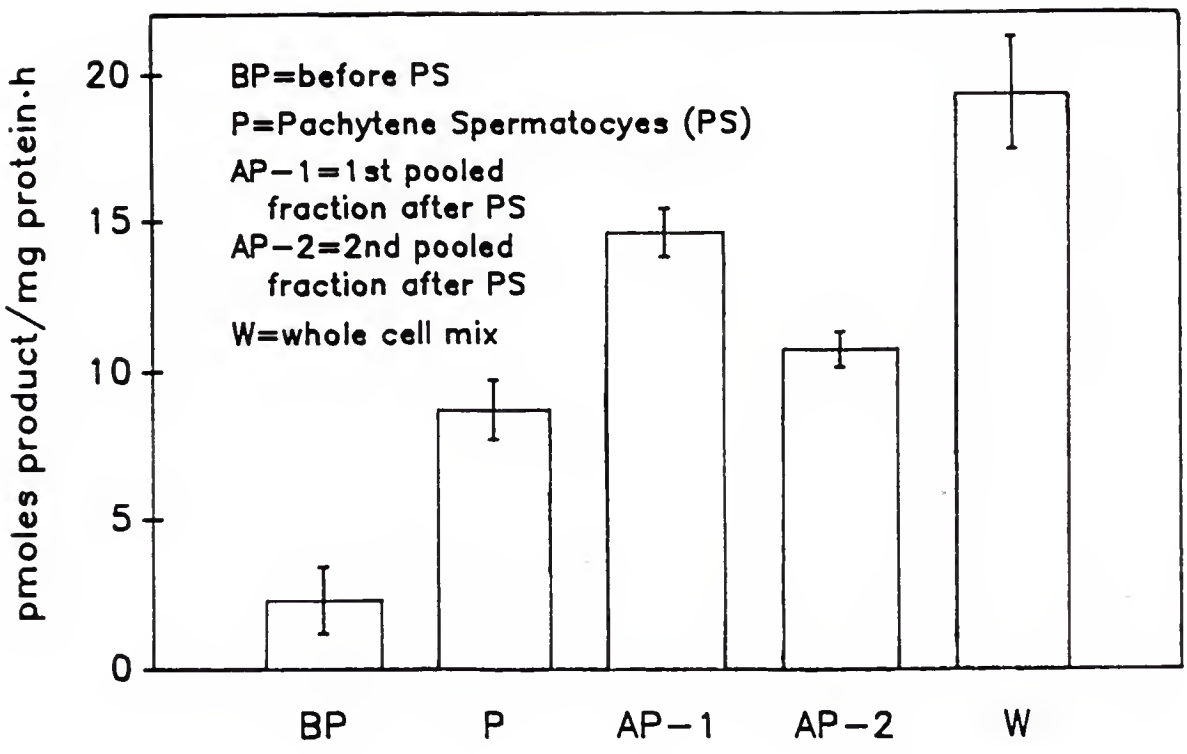

pmoles product/mg protein·h
2.0
1.5
1.0
0.5
0
P
R
23P
23W
SZ

Figure 2-15. PFT Activity Assayed in Pooled Sta Put Fractions of Spermatogenic Cells from 23 Day Old Rats.

All fractions were pooled as indicated and assayed for PFT activity with 25 μg of cytosolic protein and 30 μM Bt-KTKCVIS. Each bar value represents duplicate assays. The data are representative of 3 experiments.


pmoles product/mg protein·h
20
15
10
5
0
BP=before PS
P=Pachytene Spermatocyes (PS)
AP−1=1st pooled fraction after PS
AP−2=2nd pooled fraction after PS
W=whole cell mix
BP
P
AP−1
AP−2
W

Discussion

Testicular protein prenyl transferases. The testicular PFT and PGGT-I activities are localized to the cytosolic fraction (Fig. 2-2) as has been described for each of the protein prenyl transferases described thus far in mammals and in yeast (41, 43, 48, 71, 76-78, 80, 82, 94-96). The prenyltransferase with the highest specific activity in cytosols was PFT, with PGGT-I 15-40 fold less active. Whereas previous reports are semi-quantitative in nature, these studies give more quantitative analysis of comparisons of the levels of PFT and PGGT-I activities. Studies with partially purified enzymes have shown 8-fold higher levels of PFT activity with recombinant $p21^{c\text{-}Ha\text{-}ras}$ than seen with PGGT-I activity, measured with $p21^{rho}$ (78). Yokoyama et al. have also observed 10-fold higher levels of activity in partially purified PFT than in partially purified PGGT-I activity measured with biotinylated peptides (77). These differences in the levels of activity of PFT and PGGT-I are consistent with reports describing efforts to use peptide-affinity column chromatography for purification of prenyl transferases. The columns were a convenient way to extensively purify PFT (71) but were ineffective for purifying PGGT-I (77). It was determined that the affinity

of PGGT-I for its peptide substrates was less than that for PFT (71, 77).

Estimated K_ms of PFT for its substrates were determined to be 14 μM for Bt-KTKCVIS and 0.2 μM for [^{3}H]-FPP, which are not significantly different from that seen in the literature (Table 2-1). The K_ms of PGGT-I for Bt-KKFFCAIL and [^{3}H]-GGPP were 18 μM and 1.7 μM, respectively, also similar to that reported in the literature (Table 2-1).

Separate activities for PFT and PGGT-I were established by the exhibited differences in substrate specificity (Fig. 2-7). The strong preference of the testicular PFT to farnesylate CaaX peptides ending in serine and not leucine is consistent with many prior studies (54, 72, 77, 78). The observed geranylgeranylation of Bt-KKFFCAIL almost certainly is a measure of PGGT-I activity since PFT, which farnesylated this peptide so poorly, is not likely to use geranylgeranyl diphosphate as an effective donor. Assignment of the prenyl transferase responsible for geranylgeranylation of the PFT substrate is more difficult to assess. Yokoyama et al. (77) showed geranylgeranylation of a PFT substrate with partially purified PFT and PGGT-I (PGT). Biotinylated -NPFREKKCAIS (a PFT substrate) was geranylgeranylated by PGGT-I to the extent of 50% of that of Bt-NPFREKKCAIL (a PGGT-I substrate). They also showed that PFT geranylgeranylated Bt-NPFREKKCAIS to some extent (6-fold less than the PGGT-I), whereas this enzyme modified Bt-

NPFREKKCAIL with geranylgeranyl to negligible amounts. Therefore, although PGGT-I alone is undoubtedly responsible for the geranylgeranylation of the Bt-KKFFCAIL peptide, PFT may be responsible for some geranylgeranylation of the Bt-KTKCVIS peptide. Several investigators have reported sequences that are farnesylated by PFT in vitro, but are found to be geranylgeranylated in vivo (72, 77). Clarke proposed that this cross-reactivity suggests the relative concentrations of acceptor proteins and the two allylic donors may help direct isoprenylation in intact cells (104).

The specificity of PFT was established by comparing the ability of peptides with different CaaX sequences to serve as substrates or inhibitors. The acetylated form of the peptide acceptor (Ac-KTKCVIS) inhibited and possibly competed with Bt-KTKCVIS for farnesylation in a concentration dependent manner. Fifty percent inhibition occurred with 15 μM Ac-KTKCVIS, which suggested the acetylated peptide bound better than the biotinylated substrate to PFT. Further demonstration of specificity was shown by the lack of inhibition by N-Ac-KKFFCAIL, the acetylated peptide corresponding to the carboxy terminal sequence specific for geranylgeranylation whereas the N-Ac-KTKCVIS peptide inhibited essentially 100%.

Age-dependent activity of protein prenyl transferases. The results show that the specific activities of the testicular protein prenyl transferases PFT and PGGT-I in the

prepuberal animal vary with animal age. It is probable then that isoprenylated proteins and protein prenyl transferases are expressed in a differentiation dependent manner and possibly a cell specific manner. The activity of another testicular prenyl transferase, dehydrodolichyl diphosphate synthase, also showed dependence on animal age (24). Synthase activity increased in early stages of differentiation during the spermatogenic process, peaking at 23 days, as shown here for the protein prenyl transferases. It was concluded that this enzyme was responsible for the increase in dolichyl phosphate measured during this period of development. Among the spermatogenic cells, the pachytene spermatocytes had the highest levels of synthase activity (25).

The advantage of assaying the cytosols from rats of varying prepuberal ages is that at different times during the first wave of germ cell differentiation, distinct stages may be examined where the spermatogenic cell types present are well-known and are unaccompanied by more differentiated cell types. A 2-3 fold peak of PFT activity occurred at 26 days, when measured with either $p21^{ras}$ or Bt-KTKCVIS (Fig. 2-8). The activity decreased similarly from 26 days to 35-50 days of age, a time when the rat reaches puberty, and remains at low levels in the adult rat (Fig. 2-9).

It was of interest to assess the cellular origin, somatic or germ, of the peak of activity in 26 day old

animals. This was tested by determining if isolated germ cells had prenyl transferase activity. A 2-3 fold age dependent peak of PFT activity was observed with isolated spermatogenic cells at 23 days of age (Fig. 2-10A). Interestingly, cytosolic activity from the spermatogenic cells of 23 day old animals was 2 fold higher than activity in cytosols from decapsulated testes isolated from the same pool of animals. This may represent a loss of connective tissue and accompanying extracellular protein when purifying the germ cells. Yet the changes in PFT activity in isolated spermatogenic cells suggests that farnesylation in germ cells is important for development at this stage of spermatogenesis.

The age dependent studies performed with PGGT-I substrates and cytosol from spermatogenic cells also showed a peak of activity at 23 days of age, which was 2-3 fold higher than that seen in 17 or 28 day old animals (Fig. 2-10B). However, the specific activity of PGGT-I at each age is approximately 25 fold less than that observed for the PFT enzyme. It appears that the geranylgeranylation of proteins with the CaaL carboxy terminus is also important in the developing germ cell at this stage of spermatogenesis. However, the low activity of PGGT-I suggested that the enzyme known to geranylgeranylate proteins with alternative cysteine containing carboxy terminal sequences might be responsible for protein geranylgeranylation.

Geranylgeranylation of proteins with non-CaaX sequences was examined with MBP-rab proteins rab 1B (-GGCC), rab 5 (-CCSN), and rab 6 (-GCSC) at these same stages of spermatogenesis. The specific activity of this enzyme was extremely low in all ages tested, 5-10 fold lower than the PGGT-I activity measured with the recombinant G25K or with Bt-KKFFCAIL. There were no significant differences in activity between ages 17 and 23 days of age, but somewhat lower activity in 60 day old animals. The differences among the levels of PFT, PGGT-I, and PGGT-II specific activity was perplexing since most of mammalian prenylated proteins are geranylgeranylated (61, 62). This apparent contradiction may be partially explained by the results presented in Chapter III. The levels of protein prenylation in vivo may be determined primarily by the amount and type of substrate proteins available, thus portraying the geranylgeranylating enzymes as having a high capacity, yet low affinity for the apoproteins.

To further examine the question of somatic or germ cell origin for the peak of prenyl transferase activity at 23 days, the Sertoli cells were assayed indirectly for age-dependent PFT activity. The Sertoli cells are indispensably involved in germ cell development and are known to exhibit cyclic activity of other enzymes (6, 8, 10, 17, 19-21, 24, 25). The Sertoli cells are difficult to isolate, due to their tight junctions and their envelopment of germ cells,

and require days of culturing to release the germ cells. The lengthy culturing period and resulting uncertainty of subsequent changes in activities coupled with the low yield of cellular protein from primary cultures rendered this experiment difficult to conduct and with the possibility of obtaining questionable results. Therefore, PFT activity in cytosols from the seminiferous epithelium (Sertoli cells and germ cells) and germ cells of different aged animals were compared (Fig. 2-11). A 2-fold rise in activity occurred in both cell populations from 8 to 23 days of age, with little decrease from 23 to 35 days. This suggests that both Sertoli cells and germ cells exhibit increased activity between 8 and 23 days of age. Sertoli cells represent 68% of the cells in 8-9 day old rat seminiferous epithelium, 58% of those at 23 days, and 38% of those at 35 days of age. Thus, Sertoli cells contribute decreasing percentage of total protein in the seminiferous epithelium during testicular maturation from 8 to 23 days of age, yet the PFT activity of the seminiferous epithelium matches the increase seen with germ cells alone. Since there was no difference in the specific activity between the seminiferous epithelium and the germ cells at any age, it is most likely that Sertoli cells have PFT specific activity similar to the germ cells and also cycle in the production of prenylated proteins in spermatogenesis.

Spermatogenic cell type differences in protein prenyl transferases. The work of Zhengwei et al. (103) quantitated the numbers of spermatogenic cells present at various days of age in the developing rat. The cell types discussed were the spermatogonia, the cell types that develop from the stem cell and that undergo mitotic divisions; the spermatocytes, the cell types that develop just after DNA synthesis in prophase of meiosis; and finally the haploid spermatids, the product of meiosis which undergo extensive morphological changes as they elongate into the mature sperm. At 9 days, when PFT activity is low, the spermatogonia are the only spermatogenic cell type present. The spermatocytes appear around day 15 and by day 23, when the PFT activity peaks, they are the most prevalent cell type present. The activity declines with the presence of the spermatids at day 25 and levels off in adult animals as the spermatids continue to accumulate in numbers. The correlation of the appearance of the spermatocytes and the rise in PFT activity indicated these cell types to be responsible for the peak in activity. Therefore, PFT activity was examined in separated pachytene spermatocytes and round spermatids. Surprisingly, the levels of activity were the same in both cell types (Fig. 2-13). This observation seems to be inconsistent with either the hypothesis that 1) the increase in activity at 23 days is due to the increase in pachytene spermatocytes or 2) the subsequent decline in activity is due to the appearance of

more mature, less active spermatids. To further test the first hypothesis, pachytene spermatocytes were isolated from 23 day old rats and analyzed for PFT activity (of course round spermatids are not yet present in 23 day old animals). These cell types had 2-fold higher activity than the adult pachytene spermatocytes. This is not too surprising since studies in prepuberal animals are typically more active than the adult. For example, Potter et al. (23) showed higher incorporation of [^{14}C]-acetate into dolichol and cholesterol in prepuberal pachytene spermatocytes than in the adult pachytene spermatocytes, as well as greater HMG CoA reductase specific activity. However, the whole spermatogenic cell population from the same 23 day old animals demonstrated even higher PFT activity (4 fold) than the adult pachytene spermatocytes. Therefore the peak in activity observed at 23 days of age can not be correlated with the pachytene spermatocytes alone. Apparently, either a cell type with high activity was eliminated in the Sta Put separation and selection procedure, or the cells are more active when present with other spermatogenic cell types. The latter seems unlikely since there is no evidence of interaction between spermatogenic cells in vivo, without the presence of Sertoli cells. The presence of a soluble stimulatory "factor" can not be ruled out, but also seems unlikely since the whole spermatogenic cell mixture, which was not subjected to the Sta Put but was subjected to the

same washes in fresh medium and is maintained at 4°C, retains high activity.

To examine whether another cell type present at 23 days of age gives rise to the increase in PFT activity, all Sta Put fractions of 23 day old spermatogenic cells were pooled and assayed for PFT activity (Fig. 2-15). The pooled fractions with the highest PFT activity contain a mixture of cell types that are slightly smaller than the pachytene spermatocytes. The cell type in this mixture responsible for the high activity is probably not spermatogonia, since these are a greatly reduced fraction of the germ cells at this time and the activity is low in 9 day old animals where the spermatogonia are the only spermatogenic cell type present. Early spermatocytes, preleptotene, leptotene, and zygotene spermatocytes may be present in this mixture, yet their first appearences at days 10-15 is represented with low levels of activity. The only possible cell type that would be smaller than the pachytene spermatocytes and present in 23 day old rats is the secondary spermatocyte, the cell type that has undergone the first division of meiosis and is preparing for the second division. This is a very short-lived cell type and remains undetected by the available means of cell identification. This cell type may also be particularly sensitive to the "irregular journey" through the Sta Put, which would explain the high activity in the whole cell population which was not exposed to the Sta Put.

It seems most probable that the peak in activity at 23 days of age and the presence of higher activity in a pooled fraction of the Sta Put is due to the increased activity of of spermatocytes. The rise of PFT activity from 9 to 23 day old rat testes was not due solely to the rise in numbers of the pachytene spermatocytes as noted for other activities that increase during this time (23, 25). Although the cell type responsible for the highest activity can not be unambiguously identified, it may be the secondary spermatocyte. Undoubtedly, the peak in PFT activity occurs just prior to, during, or just subsequent to the events of the meiotic divisions in the development of the rat sperm, implicating the involvement of prenylated proteins at this point in spermatogenesis. PFT activity in spermatozoa is very low. This is not surprising, since development of the mature sperm involves stripping the cell of most of its cytoplasm.

The same cell types (adult pachytene spermatocytes, round spermatids, 23 day old pachytene spermatocytes, whole germ cell population from 23 day old animals, and spermatozoa) were found to have no significant differences in PGGT-I activity. This may be due to variable determinations for PGGT-I activity with extremely low levels of enzyme activity, which are accompanied by high backgrounds that might obliterate any potential differences. Therefore, the information gathered on the activity

differences in the isolated cell types for PGGT-I is somewhat limited.

Less can be said about prenyl transferase activities in the somatic cell, the Sertoli cell. Sertoli cells respond in a biochemically different manner to the spermatogenic cells with which they are in contact (105), so the observed increase in Sertoli cell PFT activity at 23 days of age may represent a response to the appearance of primary spermatocytes. Further work in this area will require the development of better methods for separating and culturing larger numbers of Sertoli cells.

An interesting correlation to this study is the work by Monesi (16) which showed that genetic inactivation and arrest of transcription was apparent in the spermatids. The accumulated cytoplasmic RNA was termed meiotic RNA, since it was transcribed in meiosis and released into the cytoplasm at diakinesis. The meiotic RNA is particularly long-lived and sustains the events of spermiogenesis. It is possible that the PFT and PGGT-I enzymes are made in spermatocytes, are long-lived, and thus carry the potential for prenylation activity in to the spermatids, regardless of the actual levels of in vivo protein prenylation. Analysis of protein prenylation in vivo is presented in Chapter III.

CHAPTER III
CHARACTERIZATION OF PRENYLATED PROTEINS IN THE RAT TESTIS AND ANALYSIS OF CHANGES IN PROTEIN PRENYLATION DURING SPERMATOGENESIS

Introduction

The testicular cell populations examined in Chapter II were observed to have developmental changes in PFT and PGGT-I activities, yet several questions remain unexplored. For example, does the level of in vivo prenylation of proteins reflect the changes observed in the in vitro levels of PFT and PGGT-I activities? Does the mode of prenylation (farnesyl or geranylgeranyl) reflect the levels of the farnesyl and geranylgeranyl transferase activities in rats of different ages and in isolated spermatogenic cells? Does the ratio of geranylgeranylated to farnesylated proteins change in testicular development? Do the types of prenylated proteins change in development and are they different between spermatogenic cell types? Elucidating the answers to these questions will be of value in understanding the regulation of prenylated protein biosynthesis during spermatogenesis in rat. It is well documented that the production of isoprenoids is highly regulated in the cell. Brown and Goldstein have reported several levels of regulation of mevalonate synthesis in the cell including

cholesterol feedback regulation of the enzymes HMG CoA synthase and HMG CoA reductase and the regulation of expression of LDL receptors. This regulation is deemed necessary to prevent the overproduction of cholesterol which is in excess in atherogenic tissues (30). Similar feedback regulation controls the expression of other enzymes of the isoprenoid biosynthetic pathway, including prenyltransferase (farnesyl diphosphate synthase) (31, 32) and squalene synthase (30). Cells also regulate the distribution of mevalonate products by shunting isoprenoids into non-sterol pathways which has been termed the flux diversion hypothesis by Faust et al. (106). The flux is regulated by the enzymes of the non-sterol pathway which have higher affinities for mevalonate-derived substrates (e.g. FPP) than the enzymes of the sterol pathway. This feature is particularly important when mevalonate is limiting (30). These enzymes include PFT, whose K_m for FPP is determined to be 0.04 μM with the purified enzyme from bovine brain (107) and <0.1 μM with purified enzyme from rat brain (75). The K_m of all-trans-GGPP synthase for FPP was determined to be 0.6 μM (26), which is significantly lower than that for cis-prenyltransferase (K_m = 5-24 μM) (29, 108) and squalene synthase (K_m = 1.0 μM) (109). This ensures that sufficient FPP and GGPP are available for protein modification.

Regulating the availability of isoprenoids for non-sterol dependent pathways during spermatogenesis was first

described by the work of Potter et al. who observed independent regulation of dolichol and cholesterol synthesis in the developing mouse testes (23). The rate of acetate incorporation into cholesterol increased from the preleptotene to prepuberal pachytene spermatocytes, then decreased in late pachytene spermatocytes to remain low throughout meiosis and in mature sperm. Dolichol synthesis increased in cells at early stages, remained high in pachytene spermatocytes and round spermatids, and dropped to low levels of incorporation in mature sperm. The activity of HMG CoA reductase mirrors the pattern of cholesterol synthesis. Therefore, in the developing stages of spermatogenesis and in isolated spermatogenic cells, even when HMG CoA reductase levels are low and cholesterol synthesis is low, there is enough isoprenoid flux for dolichol synthesis and probably for the prenylation of proteins in vivo.

The availability of mevinolin, a potent competitive inhibitor of HMG CoA reductase, has permitted the evaluation of isoprenoid addition to proteins. Schmidt et al. found that when radioactive mevalonate was added to the cultures of mevinolin treated Swiss 3T3 cells, proteins of 13-58 kDa were labelled with mevalonate metabolic products (48). Several investigators have noted that concentrations of 1-25 μM mevinolin (also called lovastatin) were sufficient to inhibit mevalonate synthesis, FPP and GGPP synthesis, and

protein prenylation (66, 110-112). Repko and Maltese observed that incubating murine erythroleukemia cells with lovastatin for as little as 1 h prior to the addition of cycloheximide and [^{3}H]-mevalonic acid rendered the prenylation of proteins cycloheximide insensitive (110). Since protein translation was not necessary for protein prenylation following lovastatin pretreatment, it was concluded that the blocking of mevalonate synthesis caused a depletion of isoprenoid groups and allowed an accumulation of non-isoprenylated substrate proteins that could be subsequently labelled with radioactive mevalonate precursor. This protocol also increases the level of protein labelling above that of non-mevinolin treated cells since depletion of endogenous mevalonate results in a high specific activity of the mevalonic acid when labelled mevalonic acid is added.

The work described in this chapter applied the principle described by Repko and Maltese where mevinolin was used to block endogenous mevalonate synthesis in primary cultures of seminiferous epithelium or of isolated spermatogenic cells before the testicular proteins were metabolically labelled with [^{3}H]-mevalonic acid. This chapter shows 1) optimization of incubation times and drug concentrations for the mevinolin treatment and [^{3}H]-mevalonic acid labelling in the testes system; 2) the analysis of the types and proportion of polyprenols released from [^{3}H]-mevalonic acid labelled proteins by methyl iodide

(MeI) and base treatment; and 3) the analysis of [^{3}H]-mevalonic acid labelled proteins as described by their molecular weight in one-dimensional (1-D) electrophoresis and position of migration on two-dimensional (2-D) gel electrophoresis. The latter two studies were performed with seminiferous epithelia from various aged prepuberal and adult rats and with isolated spermatogenic cells. Differences in the ratios of protein associated geranylgeraniol to farnesol (GG/F) in the seminiferous epithelium with age, and between pachytene spermatocytes and round spermatids are discussed in relation to the electrophoretic patterns of in vivo labelled proteins. The contrast of these structural changes with the changes in enzymatic activity observed in Chapter II are discussed.

Materials and Methods

Materials

Mevinolin was kindly provided by A.W. Alberts, Merck, Sharp, and Dohme, Rahway, N.J. [^{3}H]-Mevalonolactone was obtained from Dupont-NEN [35 Ci/mmol] or American Radiolabelled Chemicals, Inc. [60 Ci/mmol]. Kodak X-OMAT AR film was used for autoradiography after preflashing with a Starblitz 200 DNX camera flash set on auto. Farnesol was obtained from Aldrich Chemical Comp. and geraniol was purchased from ICN. Geranylgeraniol was a kind gift from Dr.

Alison Joly at UCLA. Electrophoresis reagents were purchased from Biorad, and all other reagents not otherwise described were obtained from Sigma Chemical Co.

Mevalonic Acid Labelling of Proteins

A suspension of cells of the seminiferous epithelium (approximately 1.5 x 10^7 cells) were incubated with 30 μM mevinolin in 0.5 ml McCoy's medium in 35 mm tissue culture dishes on a rocking platform at 32°C with 95%O_2/5%CO_2 for 3 h. [^{3}H]-Mevalonolactone (50 μCi) in 5μl ethanol was then added and the incubation continued for an additional 3 h. The cells were harvested by centrifugation and washed three times with 3 ml of PBS. The lipids were removed by three extractions with $CHCl_3$: MeOH (2:1), the $CHCl_3$ layers pooled, evaporated to dryness and stored at -20°C. The radiolabelled proteins in the aqueous phase were precipitated with 60% acetone.

Estimation of [^{3}H]-Mevalonic Acid Incorporation into Cholesterol and Dolichol

Total cellular lipid extracted from [^{3}H]-mevalonic acid labeled cells was resuspended in 1 ml 0.25% pyrogallol in methanol, 1 ml H_2O, 0.5 ml 60% KOH and saponified (113). Non-saponifiable lipids were separated by three $CHCl_3$:MeOH (2:1) extractions. This mixture was passed through a C_{18} Sep Pak cartridge (Waters) and fractions collected according to Elmberger et al. (113). The first fraction which included

the pass-through and a 10 ml methanol eluate contained cholesterol. A second fraction containing dolichol was collected with a 10 ml $CHCl_3$:MeOH (2:1) wash. Each fraction was evaporated to dryness, solubilized in 5 ml of Ready Protein+ scintillation cocktail (Beckman) and analyzed for radioactivity. Validation of the chromatographic separation of the desired products was performed with dolichol and cholesterol standards.

Estimation of Prenyl Chain Length

The prenyl groups were cleaved from proteins using a modification of the methyl iodide procedure of Casey et al. (39). Acetone precipitates of ^{3}H-mevalonate labelled proteins or mascerated 1-D gel slices (3 x 15 mm, soaked in 0.5% formic acid) of certain molecular weight regions from 1-D gels were suspended in 600 μl of 0.5% formic acid containing 10 μg of farnesol. Methyl iodide (100 μl, Aldrich) was added and the suspension incubated in darkness at 37°C for 48 h with constant stirring. Base hydrolysis was achieved by the addition of 30 μl of 35% Na_2CO_3 and incubation for 16 h at 37°C. The reaction mixture was then extracted three times with 1.6 ml $CHCl_3$: MeOH (9:1). The $CHCl_3$ layers were pooled, dried under N_2 and resuspended in 50 μl MeOH. This extract was applied to a reverse phase TLC plate (KC_8, Whatman) alongside 10 μg each of squalene, geranylgeraniol, farnesol and geraniol, then developed with

$CH_3CN:H_2O$ (9:1). One-cm sections were scraped from the plate and analyzed for radioactivity in 5 ml of scintillation cocktail. The standards were detected with anisaldehyde:H_2SO_4:95% propanol (0.5:0.5:9) (114) and the position of migration of the radiolabelled product correlated with the standard polyprenols.

One and Two Dimensional PAGE Analysis of Proteins

The acetone precipitated proteins recovered from 1.5 x 10^7 cells were suspended in 120 μl of 100 μM dithiothreitol and sonicated with several 15 s bursts. Solubilization was achieved by the addition of 230 μl of sample buffer (final concentrations: 0.55% SDS, 0.18% CHAPS, 78 μg/ml DNase I, 39 μg/ml RNase and 8.7 mM PMSF). This solution was heated at 70°C for 20 min followed by the addition of 256 mg urea. The solubilized protein (300-500 μg) was subjected to either one dimensional (1-D) electrophoresis in a 10% Tris-tricine-SDS gel (115) or isoelectric focusing by a modification of the method of O'Farrell (116). For the former, the sample was mixed 1:1 with Laemmli sample buffer (102) prior to electrophoresis. For the latter, the sample was applied directly to the isoelectric focusing gel containing 3.5% CHAPS, 2.8% pH 5-7 ampholines and 0.7% pH 3.5-10 ampholines in 3 x 115 mm tubes. Focusing was carried out for 18 h at 300 V followed by 1 h at 450 V. The extruded tube gel from isoelectric focusing was layered on the stacking gel of a

10% Tris-tricine-SDS gel for SDS PAGE (115). Molecular weight standards (Biorad) were subjected to electrophoresis simultaneously in an adjacent well. After staining the protein with Coomassie Brilliant Blue R250, the gels were soaked in 1 M sodium salicylate for 30 min, dried and subjected to autoradiography, using preflashed X-OMAT film at -85 °C. Densitometry was performed with a BioImage video image processor. The intensity of the exposure over a given area is represented as II (integrated intensity) and is in the linear range of the film and the densitometer. Alternatively, vertical lanes of Coomassie blue stained 1-D gels were sliced into 3 x 15 mm slices, each slice placed in a vial with 250 μl of 30% H_2O_2 and heated at 60°C for 48 h to digest the gel. The resulting solution was mixed with 250 μl H_2O and 10 ml of a solution of 67% Scintverse II (Fisher) and 33% Triton X-100, then analyzed for radioactivity.

Results

Optimization of labelling of proteins of the seminiferous epithelium with [^{3}H]-mevalonic acid. Before examining testicular cells for incorporation of mevalonic acid, it was necessary to first demonstrate that the cells would be viable for an extended period of time in primary culture. Seminiferous epithelium was isolated as described

in the methods of Chapter II and the viability of the cells tested by trypan blue exclusion. At the time of isolation (0 h) the cells were 99% viable. They were 98% viable at 6 h, 91% at 16h, and 76% at 24 h. Thus, seminiferous epithelium retains a viability of greater than 90% in culture up to 16 h.

Preincubation of cells with mevinolin decreases the endogenous pool of mevalonic acid and therefore enhances metabolic labelling of cellular proteins with exogenous [^{3}H]-mevalonate. The effect of varying mevinolin concentrations from 0-30 μM on protein labelling was tested by preincubating cells for 3 h with mevinolin followed by the addition of 50 μCi of [^{3}H]-mevalonic acid (final 2.86 μM) and subsequent incubation for 3 h. The cells were harvested, washed and extracted three times with $CHCl_3$:MeOH (2:1) to remove the lipids. The cellular protein was precipitated with acetone, resolubilized as described in the methods and analyzed for protein concentration and radioactivity. This labelling protocol gave 62,276 dpm/mg protein for the cells incubated with 0 μM mevinolin, 104,128 dpm/mg with 5 μM mevinolin, 51,746 dpm/mg with 10 μM mevinolin, and 177,596 dpm/mg with 30 μM mevinolin. Preincubation of seminiferous epithelium with 30 μM mevinolin increased protein labelling nearly 3-fold, therefore, subsequent studies for labelling of testicular cell proteins used 30 μM mevinolin.

The exogenous concentration of [^{3}H]-mevalonic acid was also varied to establish the best conditions for protein labelling. Cells of the seminiferous epithelium were incubated with 30 μM mevinolin for 3 h followed by a 3 h incubation with 30 μM mevinolin and four different [^{3}H]-mevalonic acid concentrations, 0.17 μM (5 μCi), 0.57 μM (10 μCi), 1.43 μM (25 μCi), or 2.86 μM (50 μCi). Protein labelling increased with increased mevalonic acid concentration: 16,724 dpm/mg for 5 μCi, 56,124 dpm/mg for 10 μCi, 118,839 dpm/mg for 25 μCi, and 221,045 dpm/mg for 50 μCi of [^{3}H]-mevalonic acid. Fifty μCi (2.86 μM) of [^{3}H]-mevalonic acid was chosen as the standard incubation condition since protein labelling was high and the expense of using higher amounts of label was avoided.

Next, a time course was performed to establish the optimal period of pretreatment with mevinolin to obtain the highest protein labelling. Seminiferous epithelium was preincubated with 30 μM mevinolin for 0-12 h prior to labelling as described above. The specific activity of labelled protein was 40,007 dpm/mg for the cells with no pretreatment with mevinolin (0 h), 86,037 dpm/mg for cells pretreated 1 h, 78,238 dpm/mg for 2 h, 84,775 dpm/mg for 3 h, and 99,640 dpm/mg for cells pretreated for 12 h. Pretreatment with 30 μM mevinolin for 1 h was sufficient for optimal incorporation of [^{3}H]-mevalonic acid into proteins,

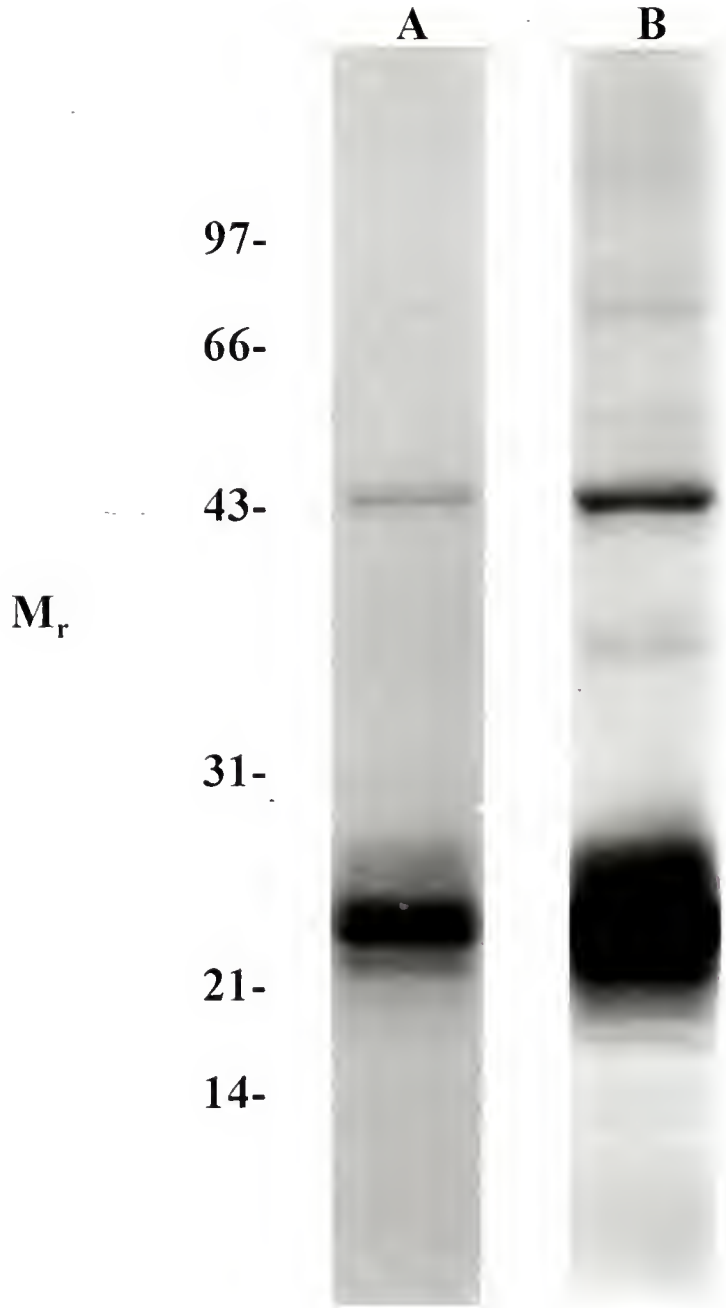
but 3 h was regularly used to ensure complete inhibition of the synthesis of endogenous mevalonate.

Spermatogenic cells exhibited equal levels of PFT activity with seminiferous epithelium (Fig. 2-2C and Fig. 2-11, Chapter II). It was of interest to determine if these cells differed from seminiferous epithelium in protein labelling with [^{3}H]-mevalonic acid. However, there was concern that the spermatogenic cells would not maintain viability in culture or take up the label in the absence of Sertoli cells. Therefore a time course of pretreatment with 30 μM mevinolin was performed with spermatogenic cells from adult rats followed by a 3 h metabolic labelling as described above. For 1 h of preincubation the proteins were found to incorporate 38,590 dpm/mg protein; for 2 h, 39,637 dpm/mg; and for 3 h, 67,618 dpm/mg. The spermatogenic cells may incorporate less radioactivity per mg protein than the seminiferous epithelium, yet good incorporation was detected within 6 h of culture.

In vivo protein prenylation. Examination of individual or groups of prenylated proteins, required that labelled testicular prenylated proteins be separated by gel electrophoresis. One-dimensional gel electrophoresis and a "short" autoradiographic exposure of mevalonate labelled proteins from the seminiferous epithelium of adult rats showed most prominent labelling in several bands in the 20-30 kDa range and in the 44 kDa range (Fig. 3-1A). Longer

Figure 3-1. One-Dimensional SDS-PAGE of [^{3}H]-Mevalonic Acid Labelled Seminiferous Epithelial Proteins.

Seminiferous epithelium was isolated from 17 day old rats, the protein labelled in vivo with [^{3}H]-mevalonic acid and 300 μg of total cell protein was subjected to SDS-PAGE as described in the methods. Autoradiography was performed for 14 days (lane A) or 30 days (lane B). The migration of molecular weight standards (x 10^{-3}) are indicated.

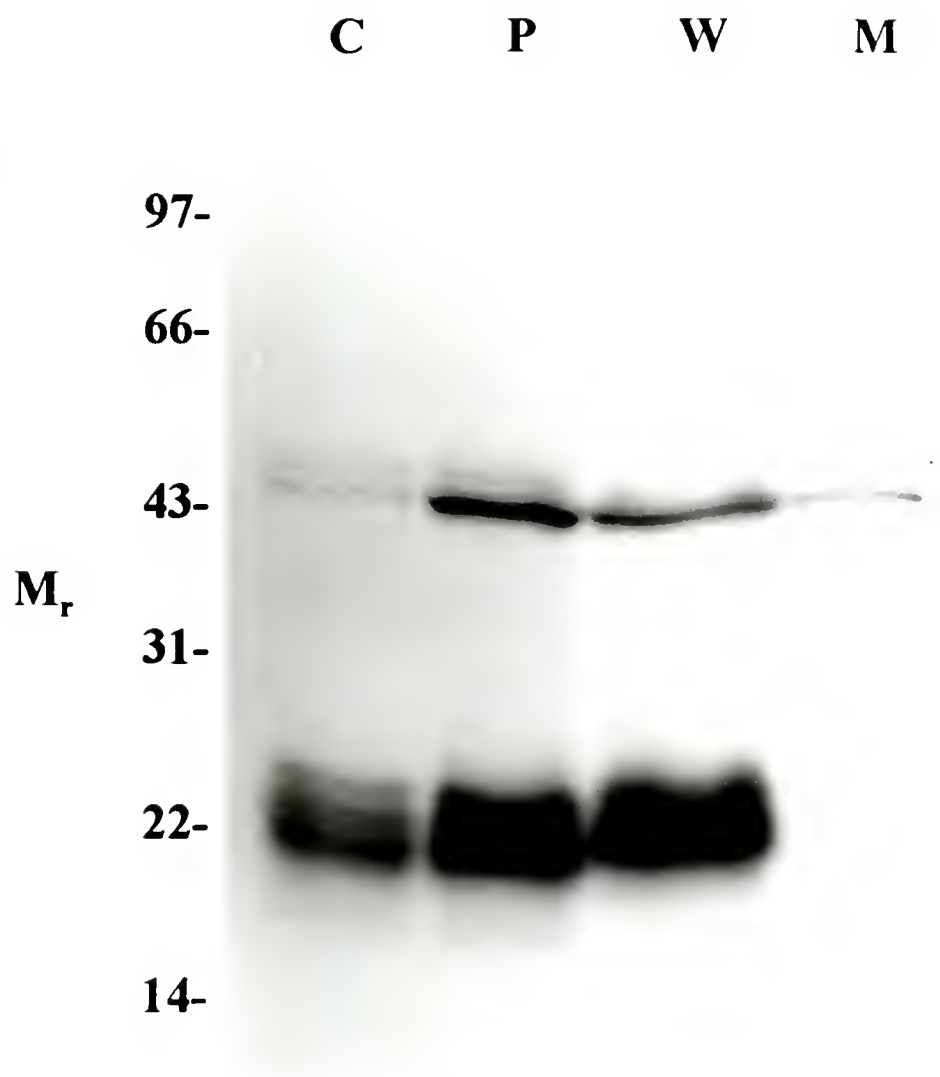

A
B
97-
66-
43-
M_r
31-
21-
14-

exposure to autoradiographic film reveals additional bands at 67, 56, 36, and 17 kDa (Fig. 3-1B).

Since the function of the prenylation of proteins was initially thought to direct the proteins to membranes (117), various subcellular fractions were examined to determine if the cellular membranes were enriched in labelled proteins. The seminiferous epithelium was labelled with [^{3}H]-mevalonate and then subjected to a crude subcellular fractionation as described in the methods of Chapter II. The seminiferous epithelium represents a mixture of morphologically different cells so membranes isolated in this way represent a varied mixture of membrane types. After centrifugation at 150-200 x g to remove whole cells (W), the supernatant (S1) was subjected to centrifugation at 100,000 x g to separate the soluble cytosolic fraction (C) from the particulate fraction (P), which contains nuclei, mitochondria, and membranes. Alternatively, the S1 supernatant was first subjected to centrifugation at 980 x g for 10 min, then 11,000 x g for 10 min to remove nuclei and mitochondria. The resultant supernatant (S2) was subjected to 100,000 x g centrifugation to yield the membrane pellet fraction (M). An autoradiogram of a one-dimensional gel of these crude subcellular fractions (Fig. 3-2) shows that there is significant amounts of label present in all fractions. The crude membrane fraction is not enriched in

Figure 3-2. One-Dimensional SDS-PAGE of [^{3}H]-Mevalonic Acid Labelled Proteins from Subcellular Fractions of Seminiferous Epithelial Cells.

Seminiferous epithelium was isolated from 48 day old rats, the protein labelled in vivo with [^{3}H]-mevalonic acid, followed by subcellular fractionation and SDS-PAGE as described in the methods. The dried gel was exposed to autoradiography for 14 days. The subcellular fractions are: C = soluble or cytosolic, P = particulate, W = whole cell, and M = membrane. The amount of protein and radioactivity loaded for each lane was: C, 200 μg and 7,200 dpm, P, 814 μg and 57,700 dpm, W, 1,210 μg and 79,800 dpm, and M, 188 μg, 4500 dpm. The migration of molecular weight standards (x 10^{-3}) are indicated.


C
P
W
M
97-
66-
43-
M_r
31-
22-
14-

prenylated proteins, yet it is interesting to note the presence of prenylated proteins in the soluble fraction (C).

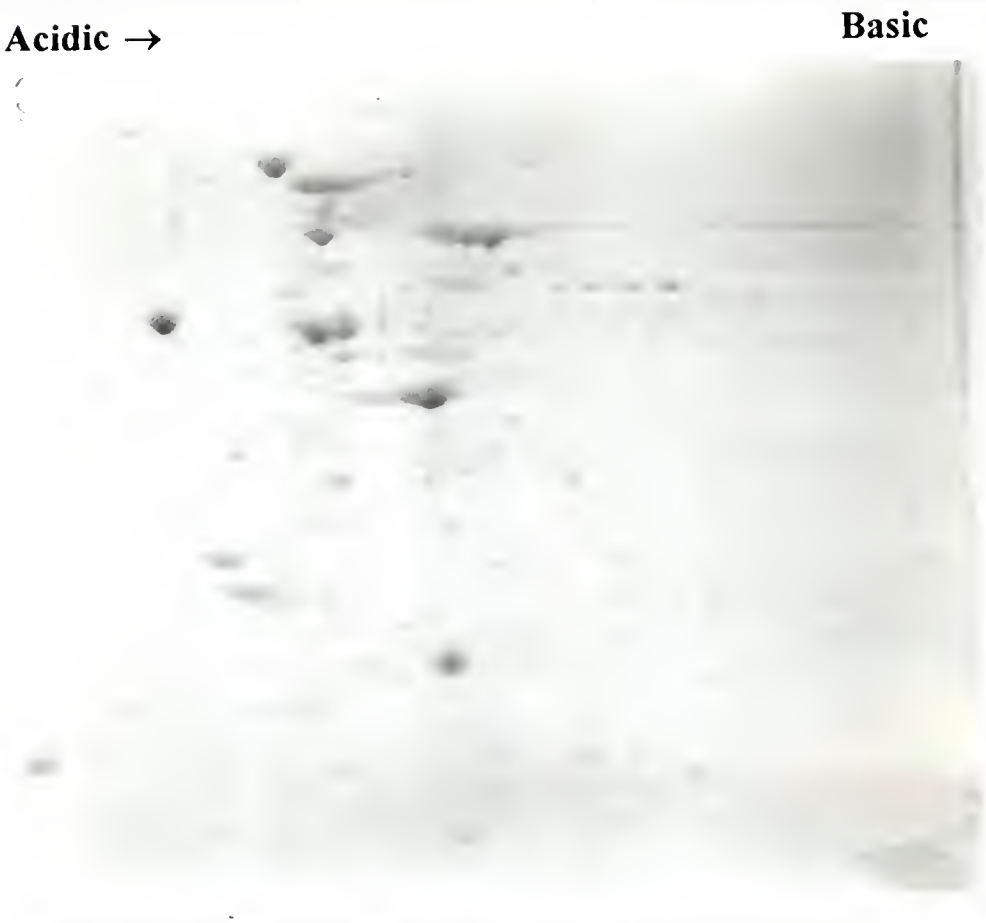
[^{3}H]-Mevalonic acid labelled protein from the seminiferous epithelium of different aged rats was subjected to two-dimensional (2-D) gel electrophoresis as described in the methods. A Coomassie blue stained 2-D gel of adult seminiferous epithelium illustrated in Fig. 3-3A shows many well defined proteins, which are also well represented at other ages. An autoradiographic exposure of a 2-D gel of labelled proteins from an adult is pictured in Fig. 3-3B. Comparisons of stained gels and autoradiograms showed that none of the radiolabelled spots correspond to stained protein, suggesting that the prenylated proteins are not highly represented. Autoradiography showed several individual proteins in the 20-30 kDa range. Many of the proteins streaked over an extended pI range, indicating that these isoprenylated proteins may be phosphorylated as described in murine lymphoma cells (118). Further analysis of the age-dependent changes in distribution of prenylated proteins will be addressed later in the chapter.

Mode of protein prenylation. The chain lengths of prenyl groups were determined on proteins of the seminiferous epithelium from the testes of different aged rats. The cells were incubated in vivo with [^{3}H]-mevalonate and the labelled polyprenols were liberated from the extracted proteins following methyl iodide treatment as

Figure 3-3. Two-Dimensional Gel Electrophoresis of [^{3}H]-Mevalonic Acid Labelled Seminiferous Epithelial Proteins.

Seminiferous epithelium was isolated from adult rats, the protein labelled in vivo with [^{3}H]-mevalonic acid followed by 2-D gel electrophoresis, as described in the methods. A 2-D gel was loaded with 200 μg of protein from a 55 day old rat and stained with Coomassie blue (Panel A). Another 2-D gel was loaded with 500 μg (162,700 dpm) of labelled protein from a 105 day old rat (Panel B) and exposed to autoradiography for 64 days. The migration of molecular weight standards (x 10^{-3}) and the orientation of pH gradient are indicated.

A



B

described in the methods. Fig. 3-4 shows the TLC separation and distribution of radiolabelled polyprenols found on proteins of the seminiferous epithelium of an adult (68 day old) rat. Isoprenoid standards squalene (S), geranylgeraniol (GG), farnesol (F), and geraniol (G) stained with iodine are also depicted. In this case, and all other conditions investigated here, GG and F were the primary and apparently only products found in the testes. These are the products observed in most other reports (119-121).

In order to identify the type of polyprenol associated with proteins of particular molecular weight classes, slices of 1-D gels containing labelled proteins were treated with methyl iodide for polyprenol analysis. A 1-D gel containing [^{3}H]-mevalonic acid labelled proteins of the seminiferous epithelium from 23 day old animals was sliced and proteins in specific molecular weight regions were analysed for polyprenol. The only labelled polyprenol retrieved from a gel slice of the 44 kDa region corresponded to the farnesol standard (Fig. 3-5A). Although the 36 kDa region contained primarily farnesol, labelled geranylgeraniol was also present (Fig. 3-5B). The 27 kDa region, the upper band of the 20-30 kDa labelled proteins, was also analysed for labelled polyprenols. Labelled geranylgeraniol and farnesol were found in almost equal amounts (Fig. 3-5C). The whole 20-30 kDa region contained mostly geranylgeraniol, approximately 3 times the amount of farnesol (Fig. 3-5D).

Figure 3-4. Thin Layer Chromatography of Polyprenols Released from [^{3}H]-Mevalonic Acid Labelled Seminiferous Epithelial Proteins.

Seminiferous epithelium was isolated from 68 day old rats, the protein labelled in vivo with [^{3}H]-mevalonic acid, followed by polyprenol analysis as described in the methods. The migration of stained isoprenoid standards (10 μg each) are depicted above (S = squalene, GG = geranylgeraniol, F = farnesol, G = geraniol). The origin (OR) and solvent front (SF) are also shown.

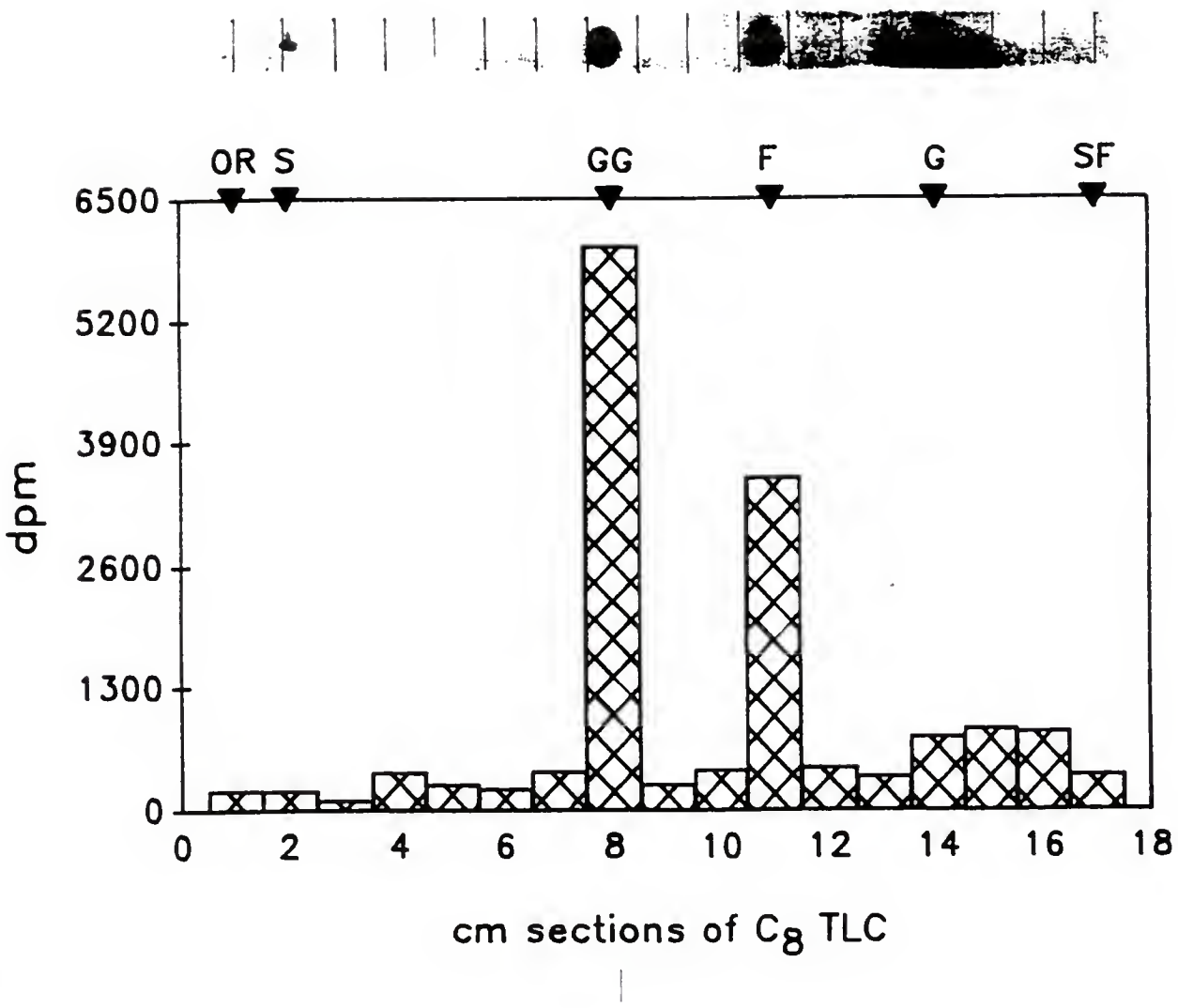
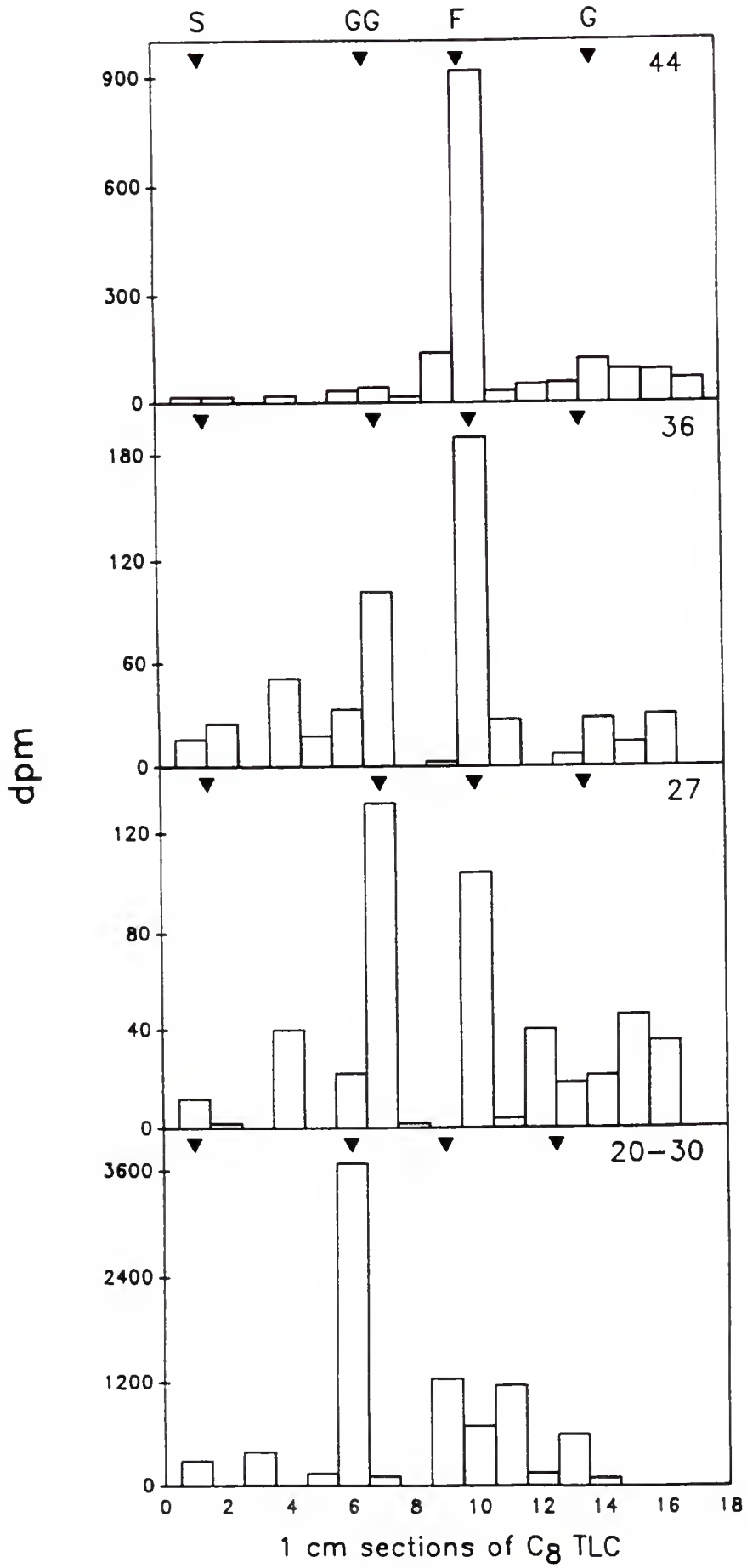

OR S
GG
F
G
SF
6500
5200
3900
2600
1300
0
dpm
0
2
4
6
8
10
12
14
16
18
cm sections of C_8 TLC

Figure 3-5. Polyprenol Analysis of [^{3}H]-Mevalonic Acid Labelled Proteins from Molecular Weight Regions of One-Dimensional SDS-PAGE.

Seminiferous epithelium was isolated from 23 day old rats, the protein labelled with [^{3}H]-mevalonic acid, followed by SDS-PAGE and polyprenol analysis of protein in gel slices from different molecular weight regions (44 = 44 kDa, 36 = 36 kDa, 27 = 27 kDa, and 20-30 = 20-30 kDa) as described in the methods. The migration of isoprenoid standards (S = squalene, GG = geranylgeraniol, F = farnesol, G = geraniol) are shown.


S
GG
F
G
44
36
27
20–30
dpm
900
600
300
0
180
120
60
0
120
80
40
0
3600
2400
1200
0
0 2 4 6 8 10 12 14 16 18
1 cm sections of C_8 TLC

Age dependence of mode of protein prenylation in seminiferous epithelium. The proportion of proteins that were geranylgeranylated verses those farnesylated was determined in rats of various ages through puberty and in the adult rat. Table 3-1 indicates a 1.6-fold drop in the ratio of geranylgeranyl to farnesyl associated with seminiferous epithelial proteins from 9 to 17 days of age. Fig. 3-6 demonstrates that the difference in ratios is largely due to a drop in the amount of geranylgeranyl with little difference in the amount of farnesyl associated with the proteins of the seminiferous epithelium. It was of interest to determine if the germ cells also changed in the proportion of geranylgeranylated to farnesylated proteins in rats of 9, 23 and 35 days of age. Therefore, spermatogenic cells and seminiferous epithelium were isolated from the same animals for each age, labelled with [^{3}H]-mevalonic acid and examined for polyprenol content. Table 3-2 shows that although the germ cells appear to be less active in labelling proteins than the seminiferous epithelium, a similar 2-fold drop of GG/F ratios occurs in both cell populations from 9 to 23 days of age. Also, in both cell populations, the drop of GG/F ratio appears to be due to a larger decrease in nmoles geranylgeranyl/mg relative to a smaller decrease in nmoles farnesyl/mg protein. The ratios in this experiment were quite low (0.4-1.5). Higher ratios

Table 3-1

Age-Dependent Ratios of Geranylgeranyl to Farnesyl Incorporated into Proteins of the Seminiferous Epithelium[a]

Age (Days)	GG[b]/F[c]
9	7.0 ± 0.1
17	4.3 ± 0.9
35	5.4 ± 1.4
73	3.7
105	4.4 ± 0.9

[a]The ratios were determined from the data depicted in Fig. 3-6.
[b]GG = geranylgeranyl
[c]F = farnesyl

Figure 3-6. Age-Dependent Differences in Polyprenols Incorporated into [^{3}H]-Mevalonic Acid Labelled Proteins of the Seminiferous Epithelium.

Seminiferous epithelium was isolated from rats of various ages through puberty and in the adult rat, the protein labelled in vivo with 2.86 μM [^{3}H]-mevalonic acid, followed by polyprenol analysis as described in the methods. The data corresponding to the standards (GG = geranylgeraniol, F = farnesol) represent the average of 2 experiments.

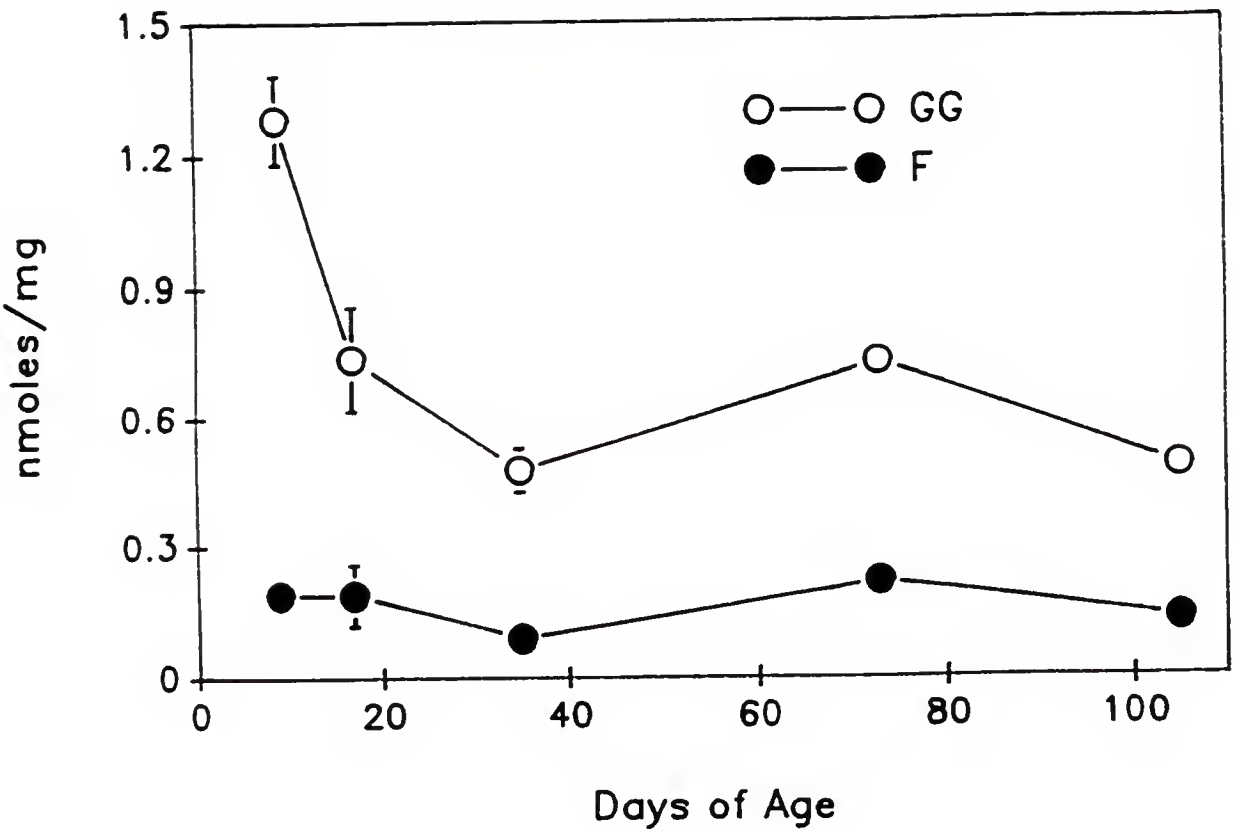

1.5
1.2
0.9
0.6
0.3
0
nmoles/mg
GG
F
0
20
40
60
80
100
Days of Age

Table 3-2

Comparison of Age-Dependent Polyprenol Incorporation into Proteins between Germ Cells and the Seminiferous Epithelium[a]

Germ Cells

Age (days)	GG[b] (nmoles/mg)	F[c] (nmoles/mg)	GG/F
9	0.33	0.29	1.14
23	0.05	0.09	0.56
35	0.21	0.50	0.42

Seminiferous Epithelium

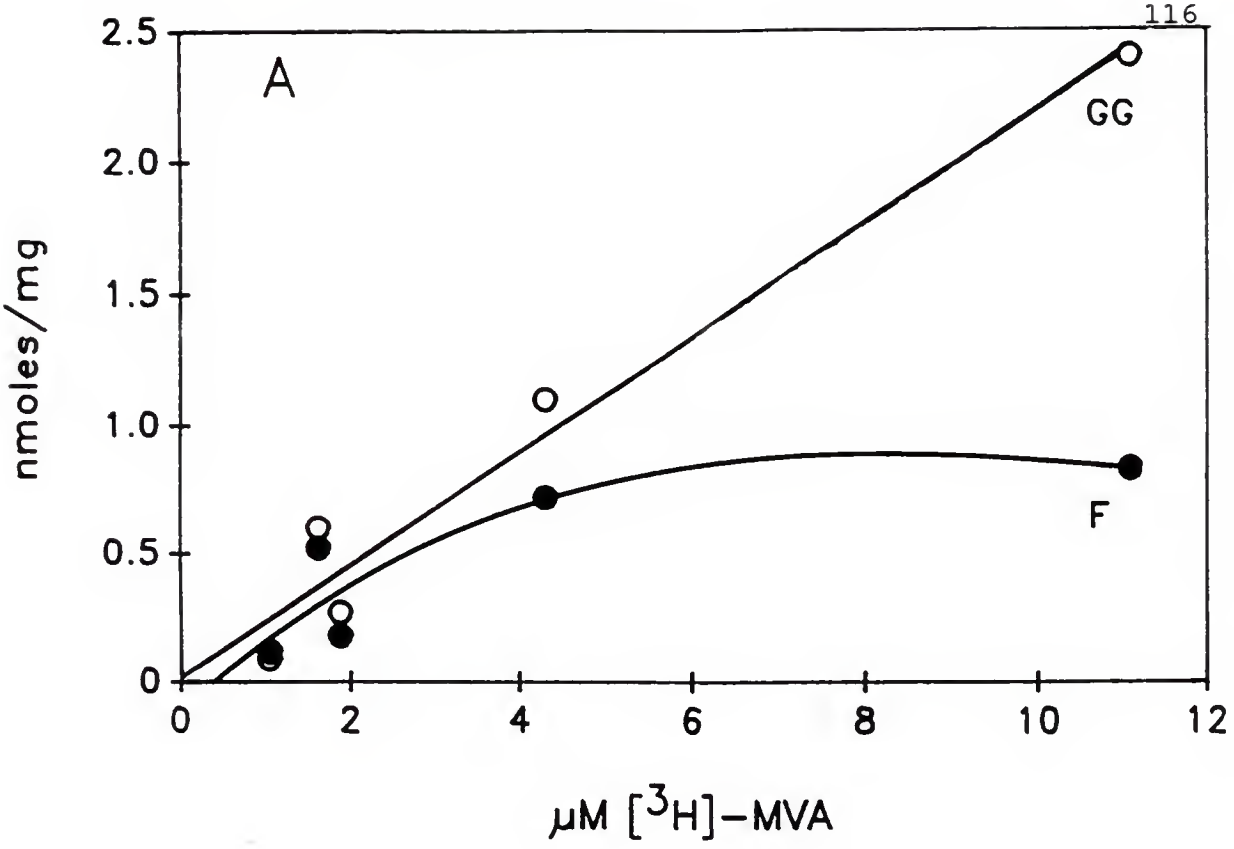
Age (days)	GG (nmoles/mg)	F (nmoles/mg)	GG/F
9	0.60	0.52	1.15
23	0.12	0.22	0.55
35	0.10	0.25	0.40

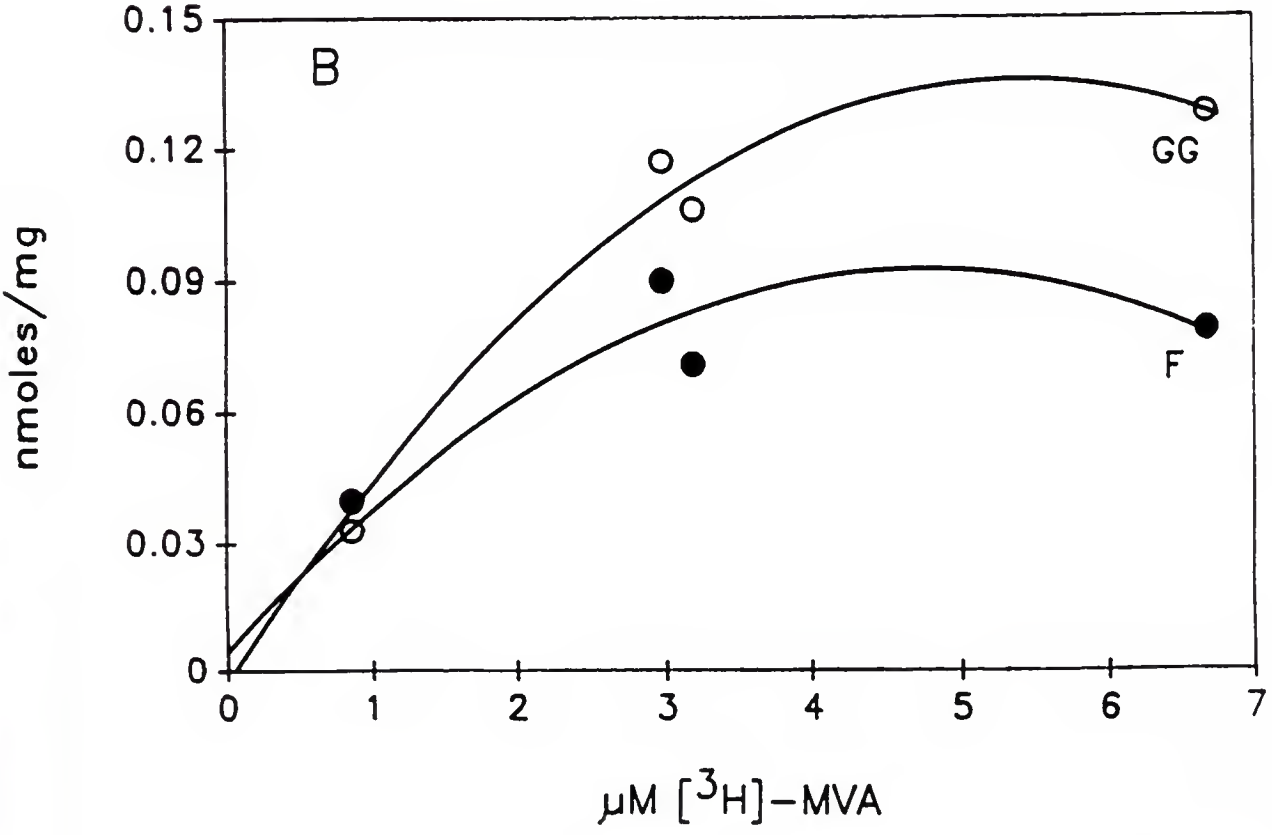
[a]Cell isolation, labelling *in vivo* with 1.6 μM [^{3}H]-mevalonic acid, and polyprenol analysis were the same as described in the methods.
[b]GG = geranylgeranyl
[c]F = farnesyl

were observed in mouse liver, kidney, brain, and lung (ratios of 4-10), as well as in tissue culture cells CHO, 3T3, HeLa, neuroblastoma, and fibrosarcoma (ratios of 4-8) (121). In addition, the GG/F values are quite different between experiments (Fig. 3-6 and Table 3-2), although the relative age-dependent differences in GG/F ratios from 9 to 17 or from 9 to 23 days of age is similar (1.6-2.0 fold) the GG/F values are quite different. Different concentrations of exogenous mevalonic acid as described in the legend of Fig. 3-6 and Table 3-2 may partially explain the discrepancy. The recent work of Rilling et al. (122) showed concentration dependence of exogenous mevalonic acid on geranylgeranyl and farnesyl incorporation into proteins of CHO cells and a concentration dependence on GG/F ratios. Therefore, the dependence of the incorporation of radiolabelled mevalonate into geranylgeranyl and farnesyl on varing concentrations of [^{3}H]-mevalonic acid was evaluated in the seminiferous epithelia of 9 and 23 day old rats (Fig. 3-7A,B). Except at very low mevalonate concentrations, <1 μM, the amount of geranylgeranyl detected was always higher than the amount of farnesyl. The incorporation of both geranylgeranyl and farnesyl into proteins in 9 and 23 day old animals increased with increasing mevalonic acid concentration. The incorporation of both isoprenoids was saturated at about 3 μM exogenous mevalonic acid in 23 day old cells (Fig. 3-7B) and in 76 day old cells (data not shown). Farnesylation was

Figure 3-7. Exogenous Mevalonic Acid Concentration Dependence of Polyprenol Incorporation into Proteins of 9 and 23 Day Old Seminiferous Epithelium.

Seminiferous epithelium was isolated from 9 day old (Panel A) or 23 day old (Panel B) rats, labelled with various concentrations of [^{3}H]-mevalonic acid and analyzed for polyprenol content, as described in the methods. The data correspond to the standards: GG = geranylgeraniol, and F = farnesol.


A
GG
F
nmoles/mg
2.5
2.0
1.5
1.0
0.5
0
0
2
4
6
8
10
12
μM [3H]-MVA


B
GG
F
nmoles/mg
0.15
0.12
0.09
0.06
0.03
0
0
1
2
3
4
5
6
7
μM [3H]-MVA

saturated at 4 μM in 9 day old cells, however, the incorporation into geranylgeranyl was still linear up to 11 μM of exogenous mevalonic acid in 9 day old cells (Fig. 3-7A). This is similar to the observations of Rilling et al. in CHO cells where farnesylation of $p21^{ras}$ was saturated at 1-2 μM, whereas increased incorporation of mevalonate into geranylgeranylated proteins occurred up to 18 μM (122). The resulting GG/F ratios increased linearly in 9 and 23 day old rats with up to 3 μM mevalonate (Fig. 3-8). The GG/F ratios for 9 day olds was 1.5-2.0 fold higher than 23 day old animals up to 3 μM of mevalonate, and appears to increase at higher mevalonate concentrations. Nine day old animals may have a larger capacity for incorporating geranylgeranyl into proteins than 23 day olds. These data suggest differences of in vivo protein geranylgeranylation versus farnesylation between ages 9 and 23 days, however, the actual levels of endogenous mevalonic acid in the testes of these animals is not known.

Dependence of mode of protein prenylation on germ cell types. Structural analysis of prenyl groups on proteins of specific testicular cells was performed on two types of spermatogenic cells, the pachytene spermatocytes and the haploid round spermatids. Following Sta Put isolation of these cells from adult rats, cellular protein was labelled with [^{3}H]-mevalonic acid and the polyprenols liberated and analyzed for radioactivity. Fig. 3-9 depicts an experiment

Figure 3-8. Exogenous Mevalonic Acid Concentration Dependence of Geranylgeranyl to Farnesyl Ratios Incorporated into Proteins of 9 and 23 Day Old Seminiferous Epithelium.

The GG/F ratios were calculated from the data shown in Fig. 3-7.

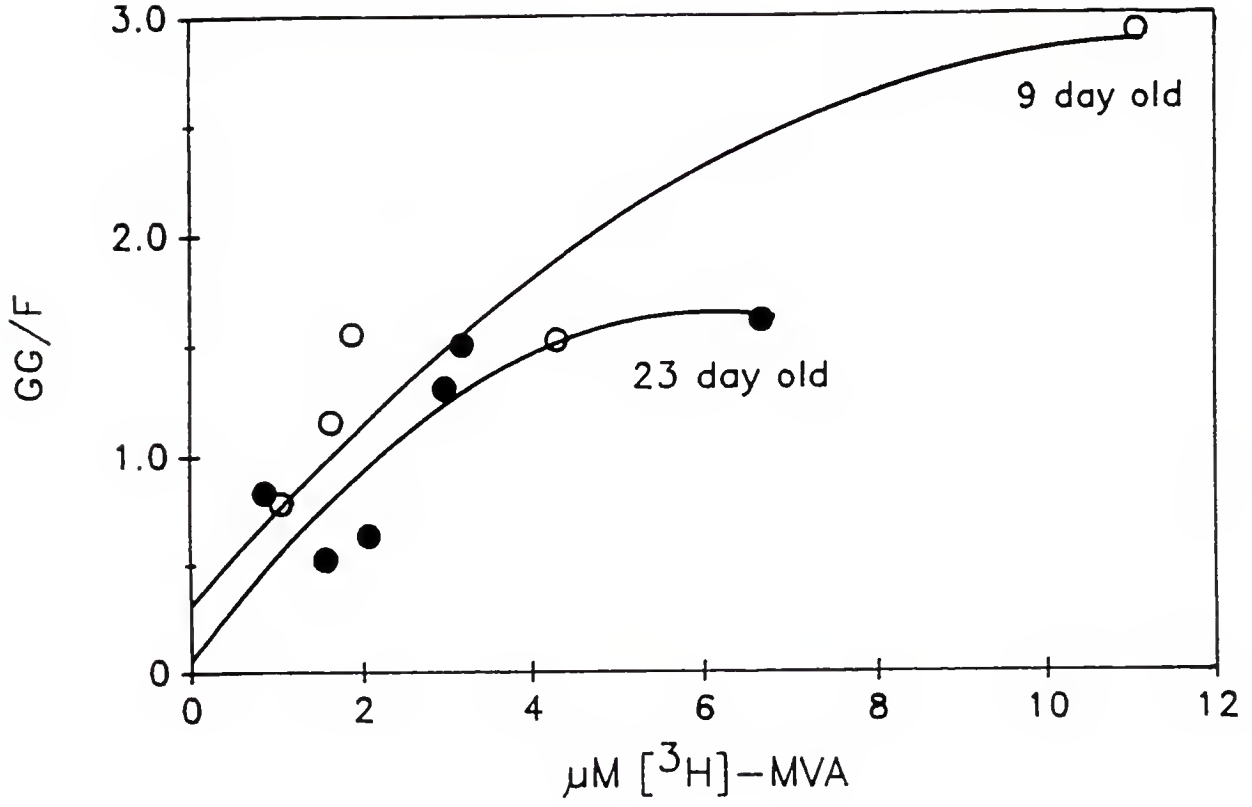
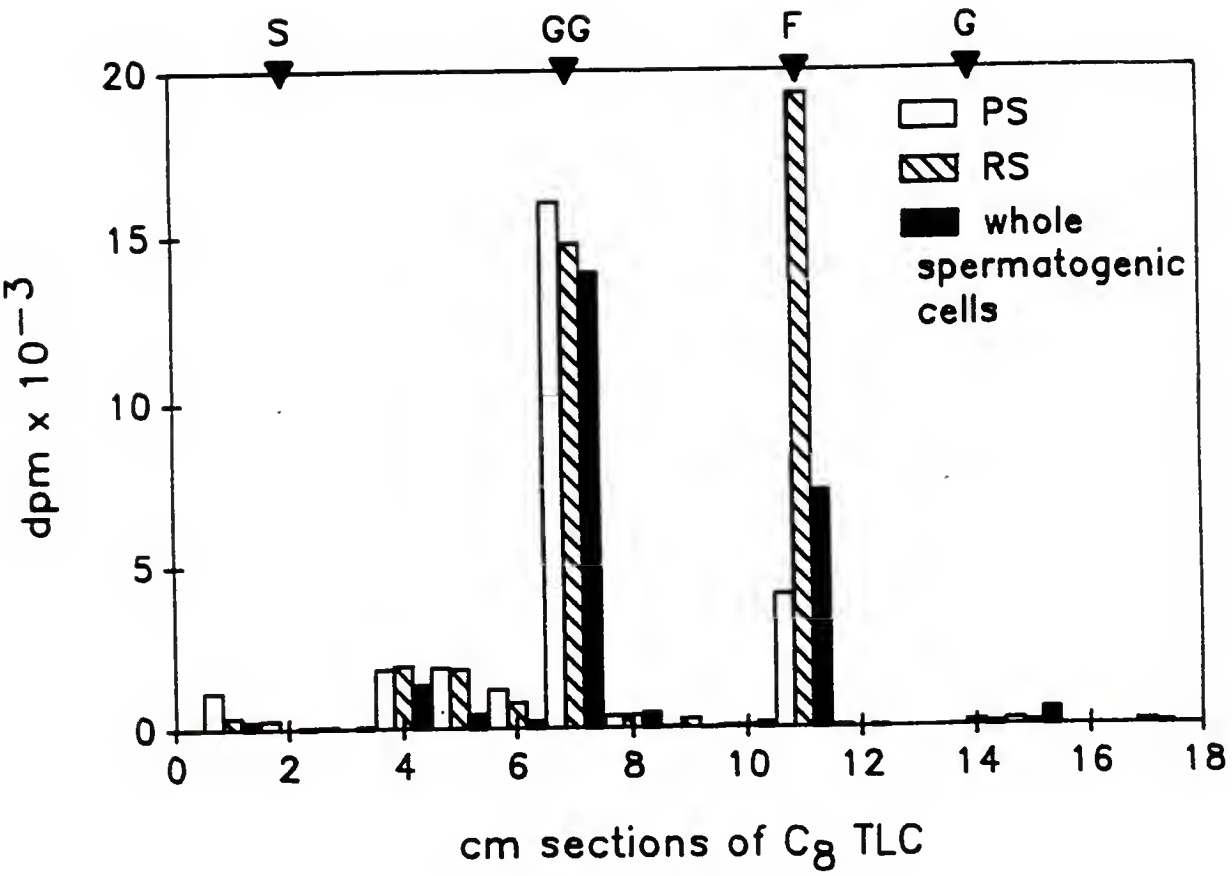

3.0
2.0
1.0
0
GG/F
0
2
4
6
8
10
12
μM [3H]–MVA
9 day old
23 day old

Figure 3-9. Thin Layer Chromatoraphy Profile of Polyprenols Released from [^{3}H]-Mevalonic Acid Labelled Protein of Pachytene Spermatocytes and Round Spermatids.

Pachytene spermatocytes (PS) and round spermatids (RS) were isolated from the whole spermatogenic cell population of a 68 day old rat, labelled with [^{3}H]-mevalonic acid and analyzed for polyprenol content, as described in the methods. The migration of isoprenoid standards (S = squalene, GG = geranylgeraniol, F = farnesol, G = geraniol) are shown. The experiment shown is representative of 3 experiments.


S
GG
F
G
20
15
10
5
0
dpm x 10^{-3}
PS
RS
whole spermatogenic cells
0
2
4
6
8
10
12
14
16
18
cm sections of C_8 TLC

comparing the TLC profiles of the labelled polyprenols from pachytene spermatocytes, round spermatids, and the whole spermatogenic cell mixture from the same adult rat. The pachytene spermatocytes have a higher proportion of geranylgeranyl than farnesyl, whereas the round spermatids have a higher proportion of farnesyl than geranylgeranyl associated with the proteins. Fig. 3-10 shows geranylgeranyl/farnesyl ratios of 3.3 ± 0.3 for the pachytene spermatocytes, 0.8 ± 0.1 for the round spermatids and 1.9 ± 0.3 for the whole spermatogenic cell mix from adult animals. The amount of labelling/mg protein for each polyprenol is depicted in Table 3-3 which indicates a greater level of labelling of pachytene spermatocytes with geranylgeranyl and a similar level of labelling with farnesyl between the two cell types.

Age dependence of in vivo protein prenylation. The relative molecular weight distribution of prenylated proteins was examined as a function of differing ages during differentiation. Dispersed cells of the seminiferous epithelium from animals aged 9-105 days were incubated with [^{3}H]-mevalonic acid and the resulting labelled testicular prenylated proteins were examined by gel electrophoresis. One-dimensional SDS-PAGE and autoradiographic analysis showed no major differences in the types (molecular weight species) of prenylated proteins, although many of the labelled proteins appeared to fade or disappear with age

Figure 3-10. Spermatogenic Cell Type-Dependent Difference in the Ratio of Geranylgeranyl to Farnesyl Incorporated into [^{3}H]-Mevalonic Acid Labelled Proteins.

Pachytene spermatocytes (PS), round spermatids (RS), and the whole spermatogenic cell population (W) were isolated from 60-70 day old rats, and analyzed for polyprenol content as described in the legend of Fig. 3-9 and in the methods. The GG/F ratios were averaged from 2 experiments.

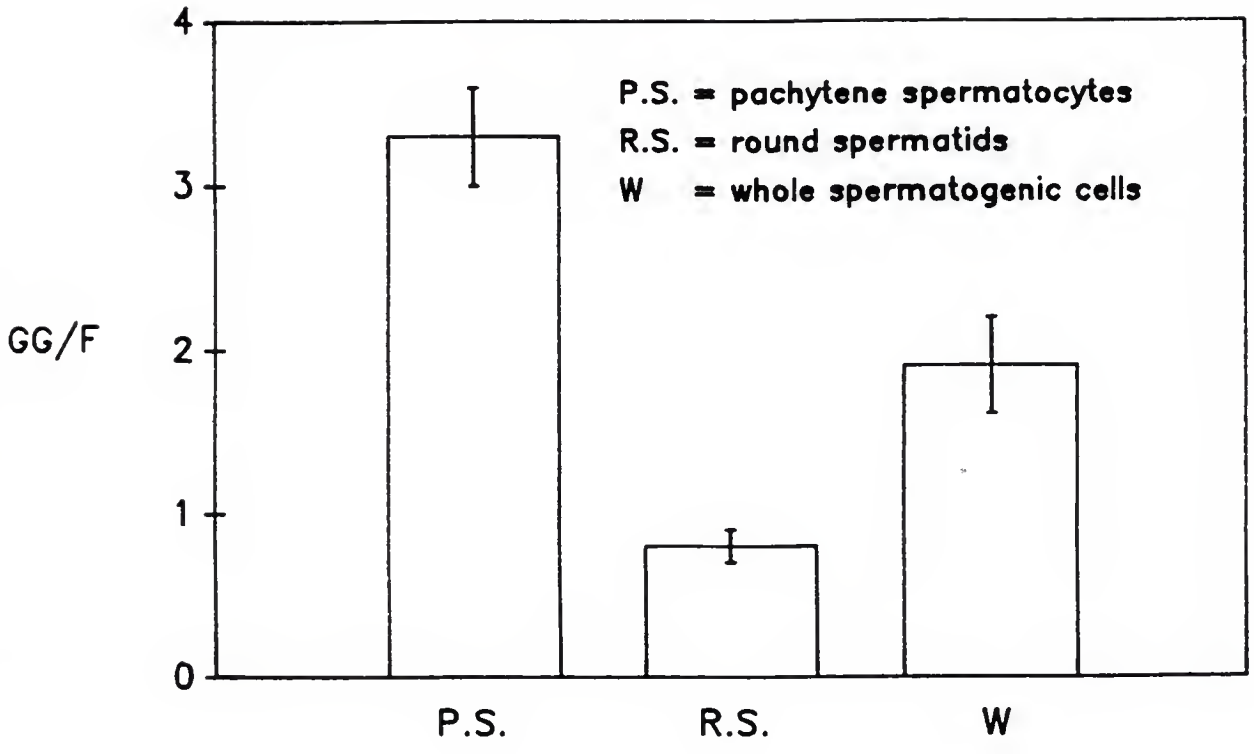

GG/F
4
3
2
1
0
P.S. = pachytene spermatocytes
R.S. = round spermatids
W = whole spermatogenic cells
P.S.
R.S.
W

Table 3-3

Comparison of Polyprenol Incorporation into Proteins between Pachytene Spermatocytes and Round Spermatids[a]

Cell Type	GG[b] (nmoles/mg)	F[c] (nmoles/mg)
Pachytene Spermatocytes	0.17 ± 0.05	0.05 ± 0.02
Round Spermatids	0.06 ± 0.01	0.07 ± 0.02
mix[d]	0.11 ± 0.004	0.06 ± 0.01

[a]Cell isolation from 70 day old rats, *in vivo* [^{3}H]-mevalonic acid labelling, and polyprenol analysis were the same as described in the methods.
[b]GG = geranylgeranyl
[c]F = farnesyl
[d]mix = the whole spermatogenic cell population from the same adult animal.

(Fig. 3-11). Densitometric analysis of each lane (age) with a shorter exposure to ensure linearity, revealed a general decrease in the amount of radiolabel per mg protein with increasing age (Fig. 3-12). The decrease was dramatic (2 fold) from 9 to 17 days of age with a slower decline from 17 to 105 days. To determine if this decrease in protein labelling was due to a decrease in the uptake of [^{3}H]-mevalonic acid, the incorporation of radiolabel into cholesterol and dolichol was measured in cells from 9, 23, and 35 day old animals. Fig. 3-13 demonstrates that there was not a decrease in the incorporation of [^{3}H]-mevalonate into cholesterol or dolichol during the time frame when the amount of label incorporated into the proteins dropped significantly.

In order to better estimate quantitative differences between labelled proteins obtained at different prepuberal ages, vertical lanes of 1-D gels, representing [^{3}H]-mevalonic acid labelled proteins from 9, 23, and 35 day old seminiferous epithelium, were sliced and analyzed for radioactivity. Relative differences in the amount of label migrating in particular molecular weight regions for 9, 23, and 35 day old animals are compared in Fig. 3-14. The most apparent differences are between the 44 and 20-30 kDa molecular weight ranges, although other subtle differences might be distinguishable. The amount of label in the 44 kDa range for 23 day old animals is 22.8% of the label in the

Figure 3-11. Age-Dependent Differences in One-Dimensional SDS-PAGE Patterns of [^{3}H]-Mevalonic Acid Labelled Seminiferous Epithelial Proteins.

Seminiferous epithelium was isolated from rats aged 9, 17, 35, and 73 days, the protein labelled with [^{3}H]-mevalonic acid, followed by 1-D SDS-PAGE as described in the methods. Three hundred μg of protein was loaded per lane, and exposure to autoradiography was for 30 days. The migration of molecular weight standards (x 10^{-3}) are indicated.

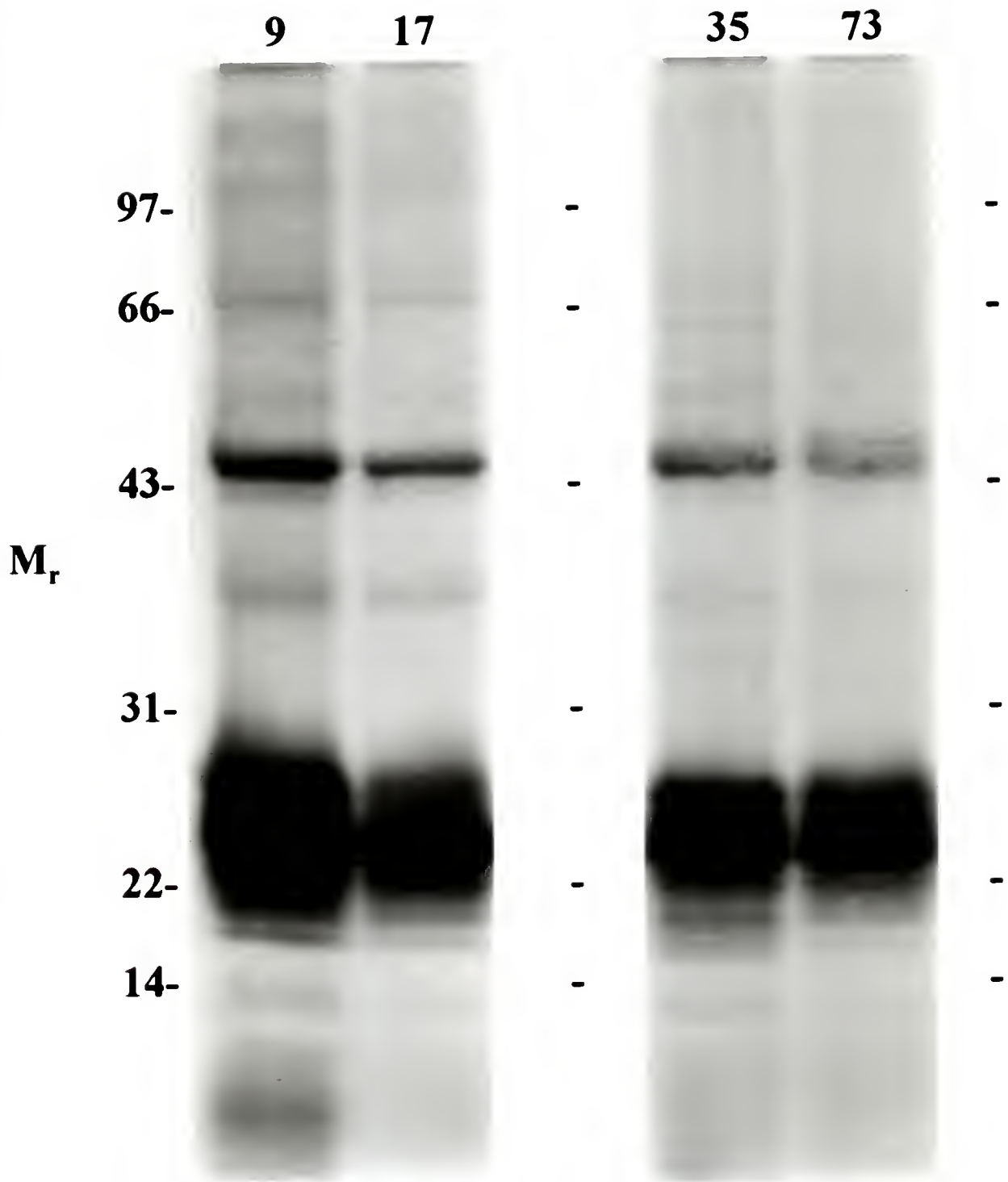

9
17
35
73
97-
66-
43-
M_r
31-
22-
14-

Figure 3-12. Age-Dependent Decrease in Specific Activity of [^{3}H]-Mevalonic Acid Labelled Seminiferous Epithelial Proteins, Quantitated by Densitometry.

[^{3}H]-Mevalonic acid labelled seminiferous epithelial proteins from rats age 9, 17, 35, 73, and 105 days were loaded 300 μg per lane on a 1-D SDS-PAGE, similar to that described in Fig. 3-11. Autoradiography was for 14 days, followed by densitometric analysis as described in the methods. The data is represented as integrated integer (II) of all exposed bands per mg protein in each lane. The data is representative of 2 experiments.

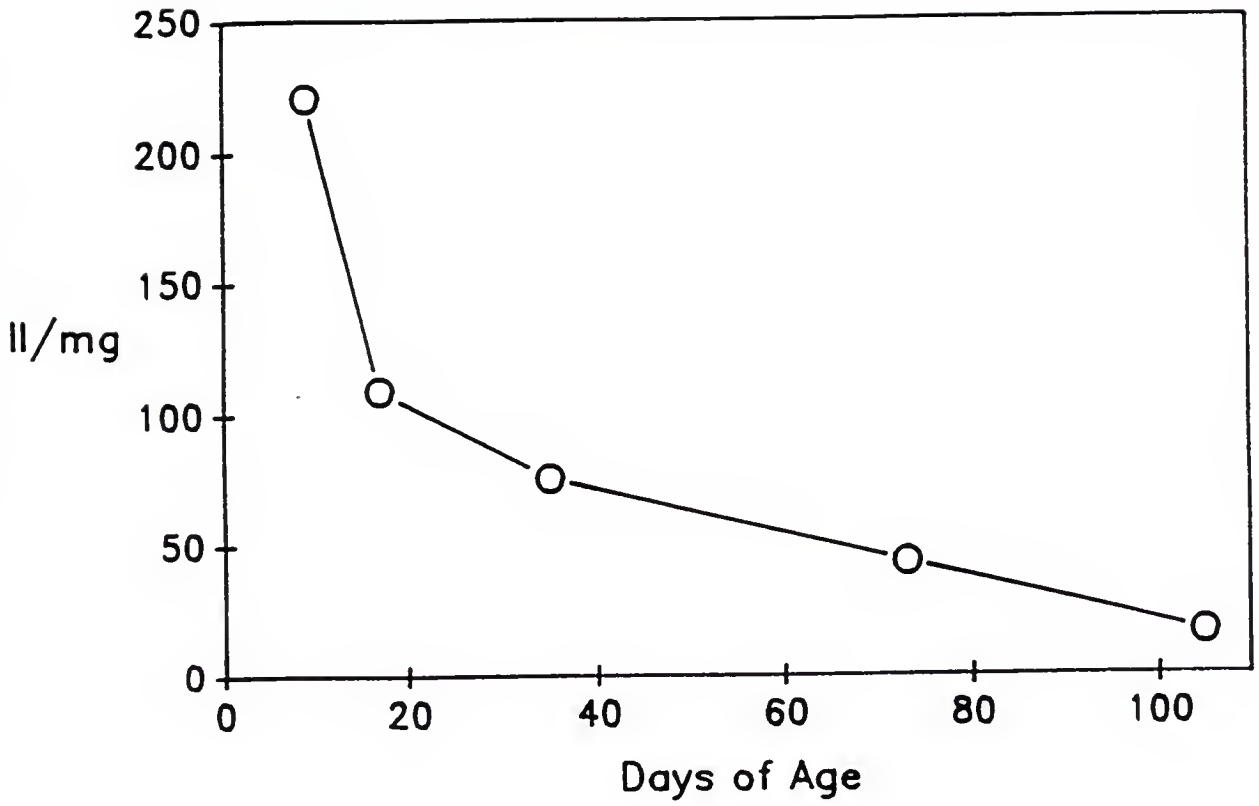

250
200
150
100
50
0
II/mg
0
20
40
60
80
100
Days of Age

Figure 3-13. Similar Levels of Incorporation of [^{3}H]-Mevalonic Acid into Cholesterol and Dolichol with Prepuberal Age.

Cholesterol and dolichol were separated from the total lipid fraction of [^{3}H]-mevalonic acid labelled seminiferous epithelium from 9, 23 and 35 day old rats, as described in the methods. The data is averaged from 3 experiments.

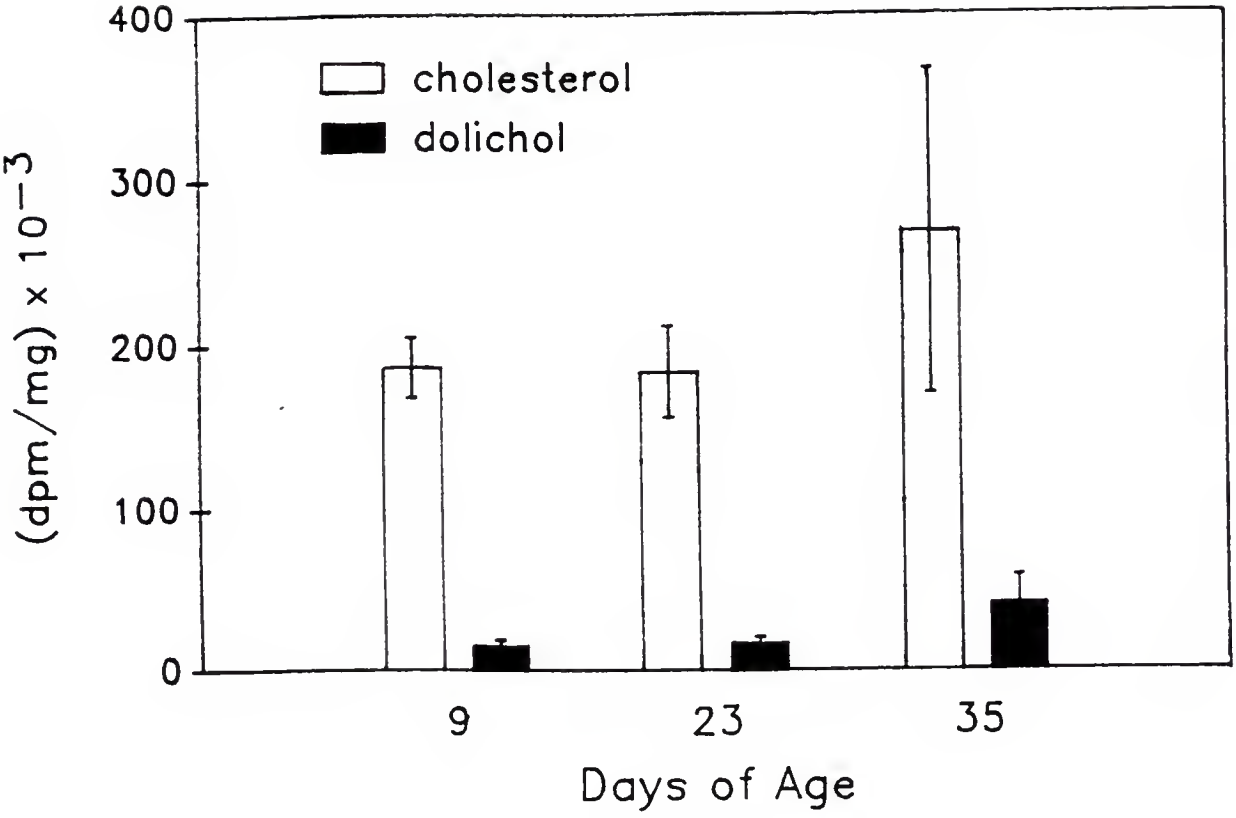
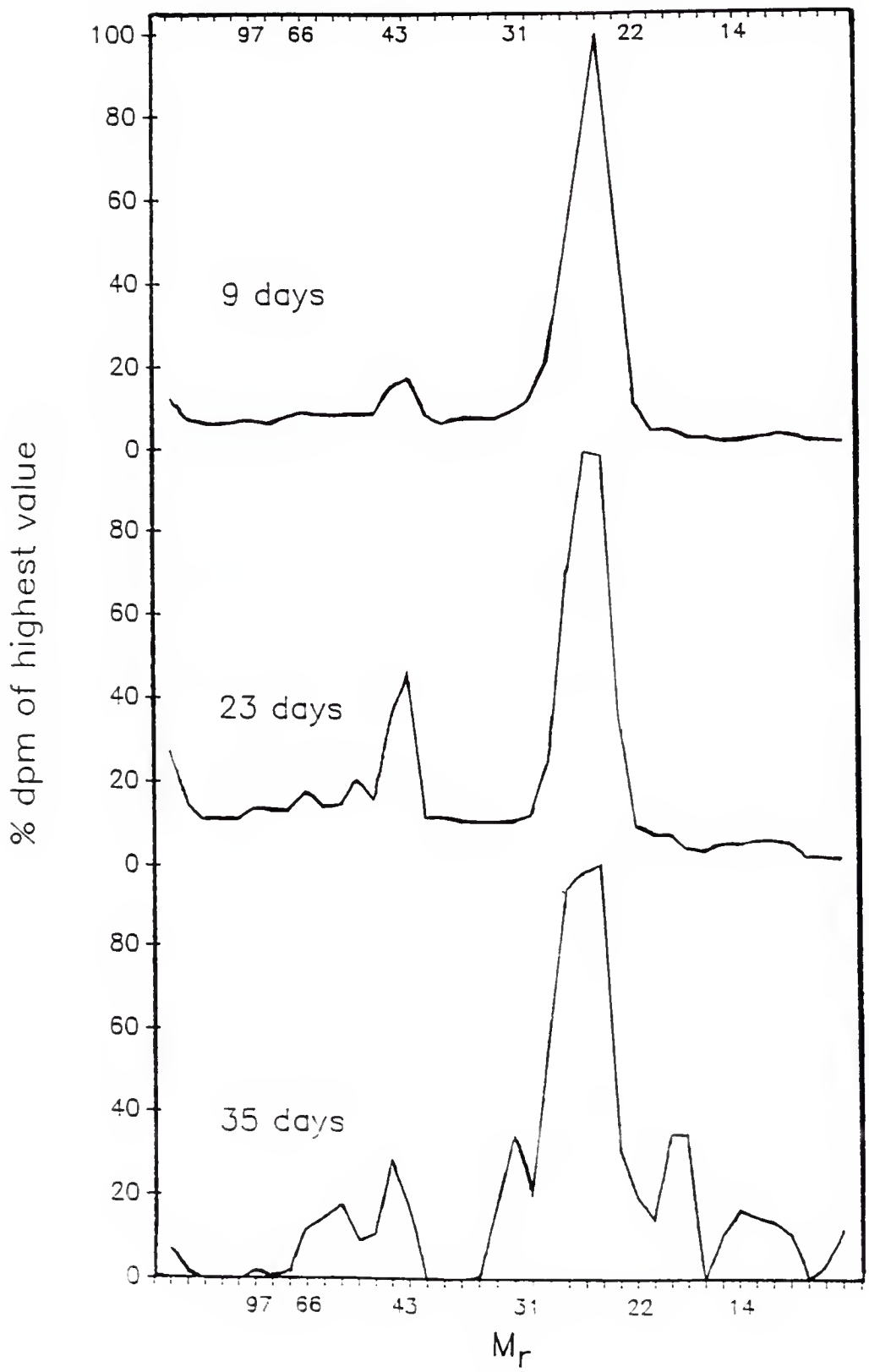

400
300
200
100
0
cholesterol
dolichol
(dpm/mg) x 10⁻³
9
23
35
Days of Age

Figure 3-14. Age-Dependent Differences in the Amount of Label in Specific Molecular Weight Regions of SDS-PAGE of [^{3}H]-Mevalonic Acid Labelled Proteins of the Seminiferous Epithelium.

Seminiferous epithelium was isolated from 9, 23 and 35 day old rats, the protein labelled with [^{3}H]-mevalonic acid, followed by SDS-PAGE and analysis of 3 mm slices for radioactivity as described in the methods. Three hundred μg of protein was loaded per lane. Radiolabel in each slice is represented as a percentage of the highest level of labelling observed for a particular age. The migration of molecular weight standards (x 10^{-3}) are indicated.

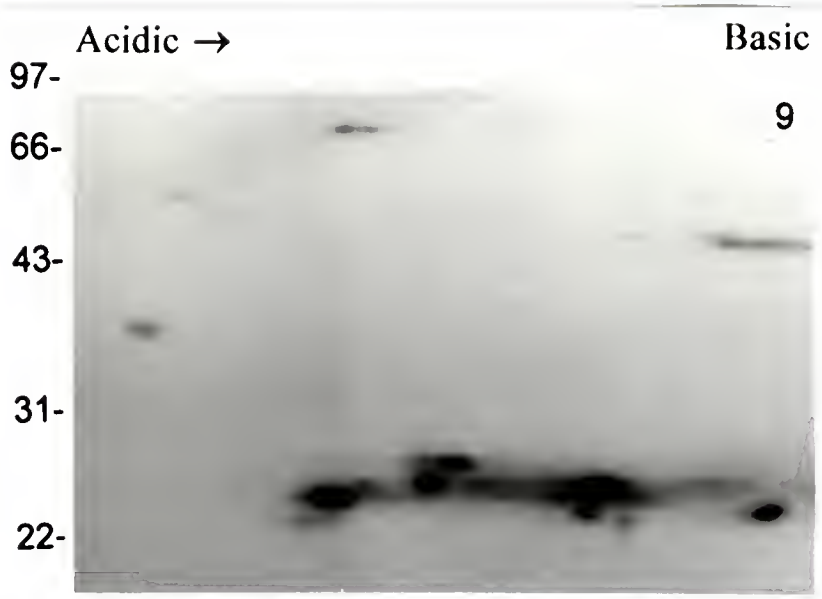

97 66 43 31 22 14
100
80
60
40
20
0
9 days
23 days
35 days
% dpm of highest value
M_r

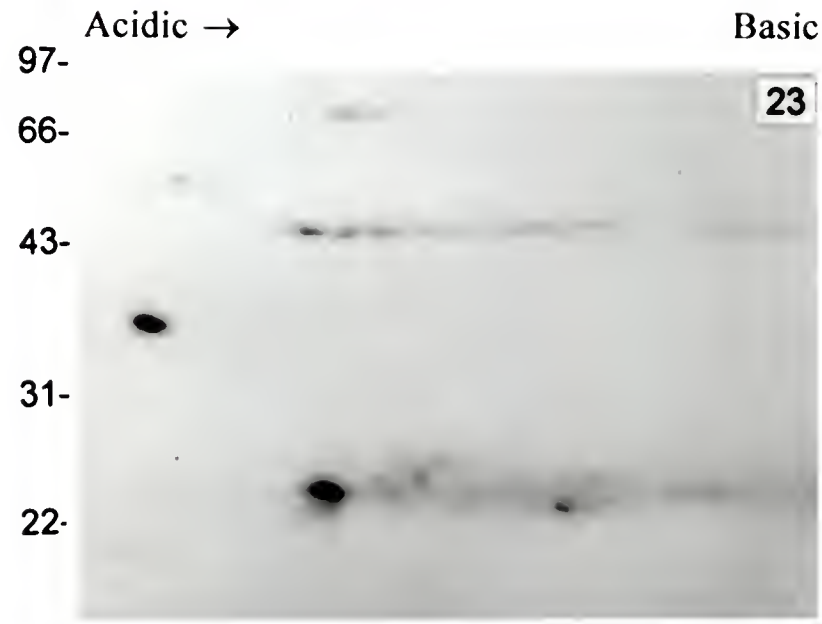
20-30 kDa range. This is in contrast to only 5.0% and 5.8% for 9 day old and 35 day old animals, respectively. Molecular weight profiles of labelled protein from 60 day old seminiferous epithelium were not significantly different than that for the 35 day old (data not shown).

Two-dimensional gel electrophoresis was employed for better separation and resolution of labelled proteins when determining age dependent differences. [^{3}H]-Mevalonic acid labelled proteins from the seminiferous epithelium of 9, 23 and 35 day old animals were subjected to 2-D gel electrophoresis (Fig. 3-15A,B,C), analyzed by autoradiography and assessed for qualitative differences in the levels of protein prenylation. Many changes occur in the prenylation of individual proteins among these prepuberal ages. Due to the decreasing levels of [^{3}H]-mevalonic acid incorporation into the proteins with age, demonstrated in Fig. 3-12, an attempt was made to load gels with equal amounts of radioactivity rather than equal amounts of protein. None of the proteins appear to remain static in the amount of label associated with them. All labelling differences in particular molecular weight proteins are described below relative to the total amount of label on the autoradiogram. These differences are intended to be qualitative and not quantitative, although semiquantitative evaluation of the intensity of several proteins relative to the total labelled protein on the gel was made by

Figure 3-15. Age-Dependent Differences in Two-Dimensional Electrophoresis Patterns of [^{3}H]-Mevalonic Acid Labelled Seminiferous Epithelial Proteins.

Seminiferous epithelium was isolated from rats aged 9, 23 and 35 days, the proteins labelled with [^{3}H]-mevalonic acid, followed by 2-D gel electrophoresis as described in the methods. The gels were loaded with 150,000-200,000 dpm and 250 μg, 440 μg, 500 μg of protein from 9, 23 and 35 day olds, respectively. The migration of molecular weight standards (x 10^{-3}) are shown and the direction of the pH gradient (approximately pH 4-7) is indicated. The autoradiograms were exposed for 60 days and are representative of at least one other experiment with identical or similar ages.


Acidic →
Basic
97-
66-
43-
31-
22-
9


Acidic →
Basic
97-
66-
43-
31-
22-
23

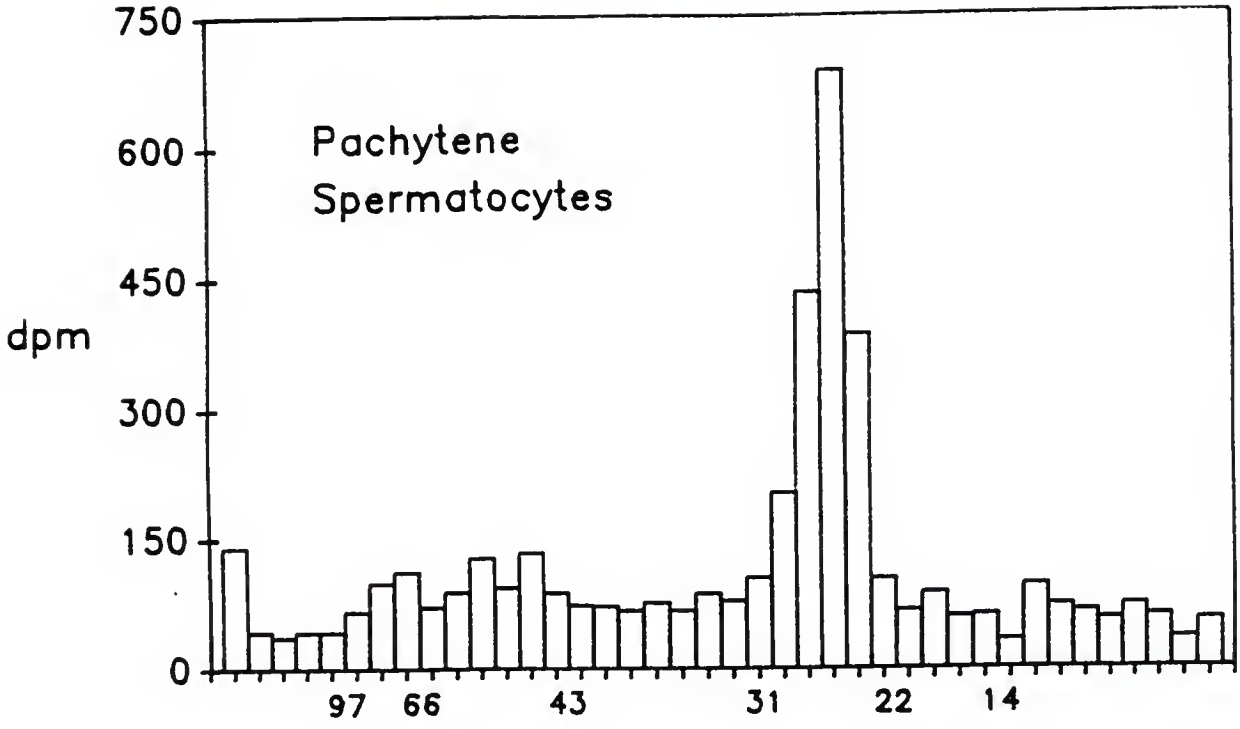

Acidic →
Basic
97-
66-
43-
31-
22-
35

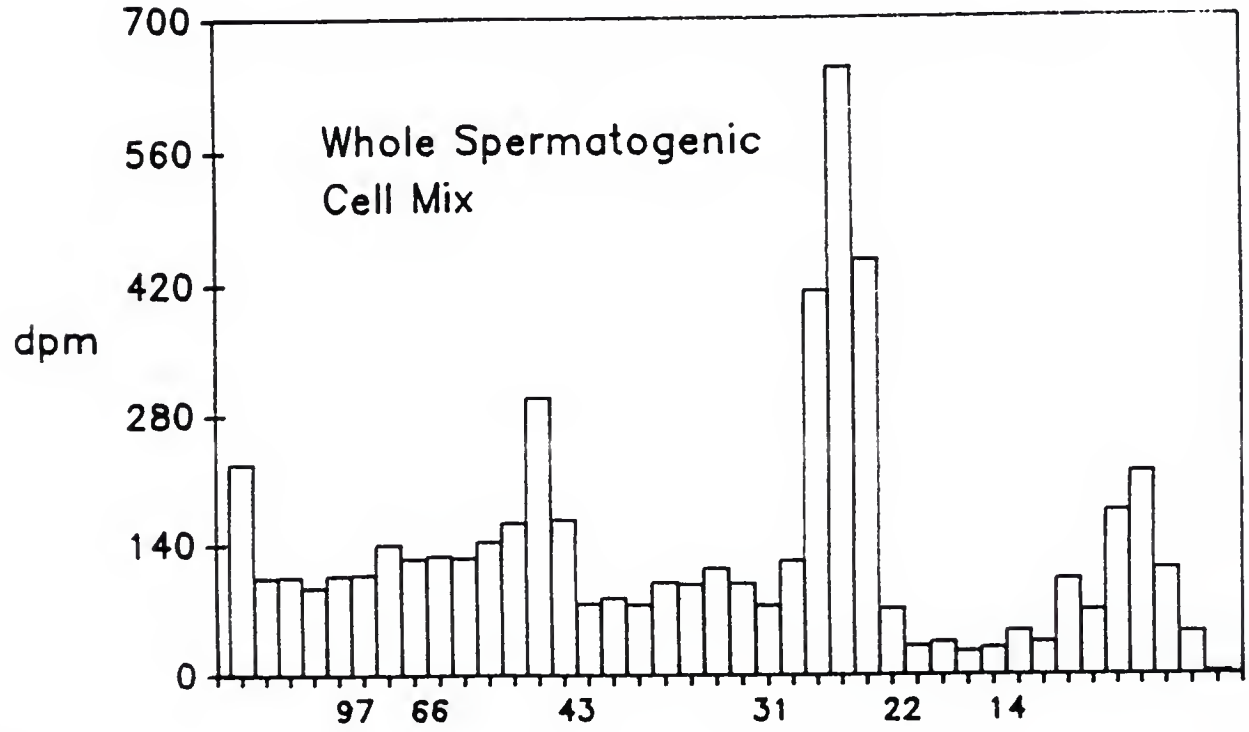
densitometry (data not shown). The most apparent change is the drop in the amount of label in the 20-30 kDa range from 9 to 23 days of age. Also prominent is the shift of label at 23 days in the 44 kDa region to a more acidic pI region of the gel. The 67 kDa protein gradually disappears while the 56 kDa protein seems to increase in the amount of label relative to the total label on the gel. The 36 kDa protein is increased in intensity at 23 days, whereas the 27.5 kDa protein, the highest molecular weight protein in the 20 kDa range, is decreased in intensity at 23 days. The most acidic 20 kDa range protein also seems to diminish markedly from 9 to 35 days of age. The age dependent changes in the amount of label associated with proteins of particular molecular weight species and correlations with the type of polyprenol associated with those proteins will be discussed below.

Spermatogenic cell type differences of in vivo protein prenylation. One-dimensional gel electrophoresis of [^{3}H]-mevalonic acid labelled proteins from pachytene spermatocytes and the whole spermatogenic cell mixture from the same adult animal is shown in Fig. 3-16. The round spermatids could not be analyzed by this method due to low levels of labelling. An obvious difference between the labelling pattern of the pachytene spermatocytes and the whole spermatogenic cell mixture is the presence of labelled 44 kDa protein(s) in the whole spermatogenic cell

Figure 3-16. Spermatogenic Cell Differences in the Amount of Label in Specific Molecular Weight Regions of an SDS-PAGE of [^{3}H]-Mevalonic Acid Labelled Proteins.

Pachytene spermatocytes and the whole spermatogenic cell population were isolated from a 68 day old rat, the protein labelled with [^{3}H]-mevalonic acid, followed by SDS-PAGE and analysis of 3 mm slices for radioactivity as described in the methods. Two hundred μg of protein and 20,000 dpm of each sample were loaded on SDS-PAGE. The migration of molecular weight standards (x 10^{-3}) are shown. The data is representative of 2 experiments.


750
600
450
300
150
0
dpm
Pachytene
Spermatocytes
97
66
43
31
22
14


700
560
420
280
140
0
dpm
Whole Spermatogenic
Cell Mix
97
66
43
31
22
14

population. The labelling pattern in the 20-30 kDa region appears to be very similar between the two cell populations.

Since low levels of labelling were observed in proteins of the round spermatids, it was suspected that differences in [^{3}H]-mevalonic acid uptake might be responsible, so round spermatids and pachytene spermatocytes were analysed for [^{3}H]-mevalonic acid incorporation into cholesterol and dolichol. Table 3-4 illustrates that the incorporation into cholesterol is similar between the two cell types, yet the incorporation into dolichol appears to be decreased in the round spermatids relative to that of the pachytene spermatocytes. This suggests that although there does not appear to be a problem with uptake of [^{3}H]-mevalonic acid into the round spermatids, there may be a shift in isoprenoid utilization in this cell type.

Finally, [^{3}H]-mevalonic acid labelled proteins from round spermatids and pachytene spermatocytes were separated by 2-D gel electrophoresis. Fig. 3-17A displays low amounts of labelling in the 44 kDa range in pachytene spermatocytes, but good labelling of 20-30 kDa proteins. The round spermatids (Fig. 3-17B), however, appear to preferentially label the 44 kDa region, implicating the 44 kDa proteins in the post-meiotic stage of spermatogenesis. In contrast, round spermatids show little labelling of the 20-30 kDa proteins.

Table 3-4

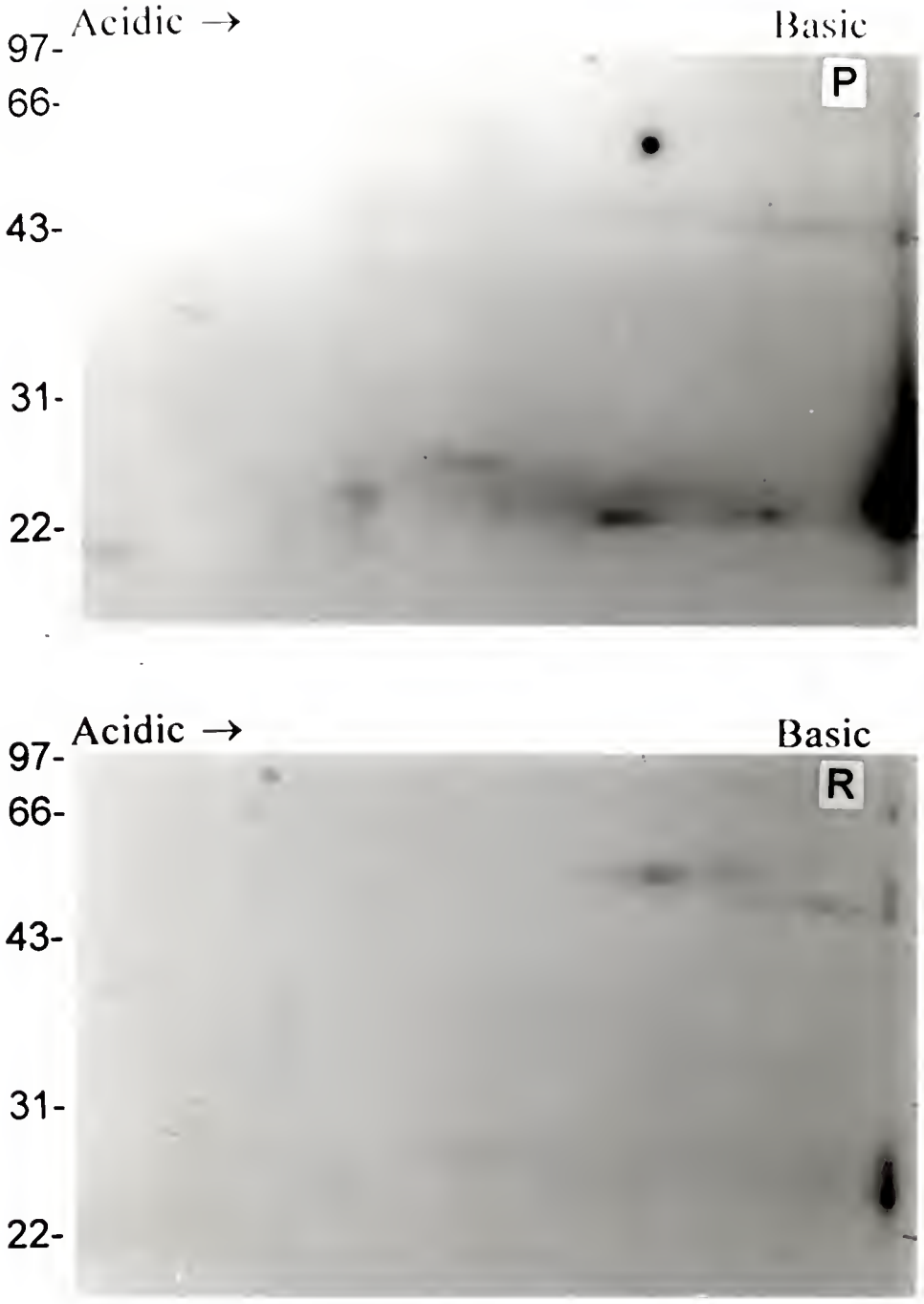
Comparison of ^{3}H-Mevalonic Acid Incorporation into Cholesterol and Dolichol between Pachytene Spermatocytes and Round Spermatids[a]

Cell Type	Cholesterol (dpm/mg)	Dolichol (dpm/mg)
Experiment 1		
Pachytene Spermatocytes	132,867	12,424
Round Spermatids	63,008	1,603
Experiment 2		
Pachytene Spermatocytes	165,465	24,146
Round Spermatids	236,917	10,700

[a]Cell isolation, *in vivo* [^{3}H]-mevalonic acid labelling, and cholesterol/dolichol analysis were the same as described in the methods.

Figure 3-17. Spermatogenic Cell Type Differences in Two-Dimensional Gel Electrophoresis Patterns of [^{3}H]-Mevalonic Acid Labelled Proteins.

Pachytene spermatocytes (P) and round spermatids (R) were isolated from spermatogenic cells of an 82 day old rat, the proteins labelled with [^{3}H]-mevalonic acid, followed by 2-D gel electrophoresis as described in the methods. The gels were loaded with: (P), 250 μg and 43,000 dpm, (R), 260 μg and 14,000 dpm. Exposure to autoradiography was for 90 days. The migration of molecular weight standards (x 10^{-3}) and the orientation of the pH gradient are indicated.


Acidic →
Basic
P
97-
66-
43-
31-
22-
Acidic →
Basic
R
97-
66-
43-
31-
22-

Discussion

In vivo protein prenylation. Metabolic labelling studies were performed with primary cultures of particular testicular cell populations by incubating with 2.86 μM (50 μCi) of [^{3}H]-mevalonic acid in the presence of 30 μM mevinolin for 3 h, following a 3 h pretreatment with 30 μM mevinolin. These conditions were found to be optimal for maximum incorporation of label into proteins. The metabolic labelling allowed examination of the types of proteins labelled and the mode of prenylation in different testicular cell populations from rats of different ages.

The types of proteins labelled are described by their molecular weights, determined by their migration on SDS-PAGE. The molecular weight pattern of prenylated proteins is similar to that seen in other tissues and cell lines (40, 48-50) with the exception of the 36 kDa protein. This protein appears to more highly labelled in the testicular system. Subcellular fractionation of [^{3}H]-mevalonic acid labelled seminiferous epithelium yields some separation of labelled proteins (Fig. 3-2) and raise speculation as to the function of protein prenylation. The particulate fraction contains labelled proteins in the 20-30 kDa region and 3 bands at 44-47 kDa. The lowest 44 kDa band contains the most radiolabel and is the most apparent of the 3 bands in whole cellular labelled protein. The membrane fraction contains

the low band at 44 kDa and almost no label anywhere else. The cytosolic fraction contains labelled proteins at 20-30 kDa, 36 kDa, 56 kDa and 3 bands at 44-47 kDa. In contrast to the particulate fraction, the 3 bands at 44-47 kDa have similar levels of radiolabel. Studies of subcellular fractionation in other cell types have been reported. Maltese and Sheridan found in neuroblastoma cells, 53 kDa, 26 kDa, 22 kDa, and 17 kDa proteins in the cytosolic fraction, 66 kDa in the nuclear fraction, 45 kDa in all fractions, and 22-26 kDa in the mitochondria/membrane fraction (49). Sepp-Lorenzino et al. found in murine lymphoma cells, 44-69 kDa proteins in the nuclear fraction, 21-24 kDa proteins in the cytosol and no proteins in mitochondria, microsomes, or plasma membrane (118). The 66 or 69 kDa protein has been identified as lamin B, which is a nuclear matrix protein (44). The observation that prenylated proteins are prevalent in the soluble as well as particulate fractions of cells indicates that earlier predictions of the prenylation of proteins as a membrane targetting modification (117) do not appear to adequately describe the function of this modification. In fact, the prenylation of $p21^{c\text{-}Ki\text{-}ras}$ and of $p21^{rhoA}$ has been found to be necessary for the interaction of these proteins with soluble stimulatory GDP/GTP exchange proteins (123). Interaction with other proteins may be a unique role for prenylated proteins in

intracellular signalling in addition to accounting for the cytosolic location of these lipid modified proteins.

Maltese et al. found that most 20-30 kDa proteins were geranylgeranylated and that the 66 kDa, 56 kDa, and 47 kDa proteins were farnesylated (112). Polyprenol analysis of labelled proteins of the seminiferous epithelium in some of these same molecular weight regions showed similar modes of prenylation of the testicular proteins. The 20-30 kDa region consists mostly of geranylgeranylated proteins, with a GG/F ratio of 3. Many of these prenylated proteins have been identified by others as $p21^{ras}$-like GTP-binding proteins (G-proteins) (104). The prenylated G-proteins in the low molecular weight region are members of the ras superfamily of genes, which include ras, ral, rac, rho, rap, and rab (124). These proteins are central components of signalling pathways that regulate transport vesicle fusion and fission (125). Although $p21^{ras}$ is farnesylated, most low molecular weight G-proteins are geranylgeranylated (104). Transcripts of ras, as well as the protein product $p21^{ras}$ have been found in testicular cells (11, 12). However, the monoclonal antibody Y13-259 did not recognize detectable amounts of the $p21^{ras}$ proteins on a western blot of seminiferous epithelial proteins, although the recombinant $p21^{H\text{-}ras}$ standard was detected (data not shown).

In an attempt to analyze a prenylated protein of the 20-30 kDa range, the band corresponding to 27 kDa on a 1-D

SDS-PAGE was sliced from the gel and found to contain both labelled geranylgeraniol and farnesol in equal amounts. The band probably represents several labelled proteins. A single protein covalently modified with geranylgeranyl or farnesyl is conceivable, but there is no evidence of an isoprenylated protein containing both types of polyprenol in vivo. The 36 kDa protein(s), however, was clearly separated from other labelled proteins, and the same procedure revealed both labelled geranylgeraniol and farnesol. The reason for this is unclear. In contrast, the proteins in the 44 kDa region only contained farnesol. These proteins may correspond to the 44 kDa protein described in murine lymphoma cells as a nuclear matrix-intermediate filament protein (118), although in the seminiferous epithelium the 44 kDa proteins were found in the cytosol and membrane fractions and were not exclusively localized to the particulate fraction containing nuclei (Fig. 3-2).

The mode of prenylation of all labelled proteins of the seminiferous epithelium was examined in rats of various ages through puberty and in the adult rat. Since PFT and PGGT-I activities increased in early ages of prepuberal rats and declined by day 35 to lower levels in adult rats, it was of interest to determine if these changes in activity were reflected in in vivo changes in the amount and type of polyprenol associated with the proteins. In primary cultures of seminiferous epithelium and spermatogenic cells, there

appears to be high levels of both polyprenol types at 9 days of age with a predominance of geranylgeraniol (GG/F = 1.2). The amount of geranylgeranyl associated with the proteins decreases 1.6-2.0 fold with a smaller decrease in farnesyl from 9 to 23 days of age. Thus, at a time when the activities of PFT and PGGT-I are increasing and the activity of PGGT-II is unchanging, the in vivo amount of total protein prenylation is decreasing.

The generally higher level of geranylgeranylation compared to farnesylation of proteins, however, is not accounted for by the measured activities of PFT, PGGT-I and PGGT-II. The low level of measured geranylgeranyl transferase activity could be accounted for by at least three factors. One, that optimal conditions for measuring PGGT-I were not achieved (e.g. the biotinylated peptide is not the optimal substrate) or two, geranylgeranyl transferase(s), as yet undescribed, with specificity for other carboxy terminal sequences are more active in this tissue. PGGT-II activity, measured with several alternative protein acceptors, gave very low activity with no age dependent variance (Table 2-2). A third possibility is that proteins available for geranylgeranylation are high in concentration at early ages of differentiation and they decrease more rapidly than proteins available for farnesylation as differentiation proceeds. Thus the higher yield of geranylgeranylated proteins in early ages would

result from a higher production of protein acceptors than in sequential ages.

Rilling et al. showed in CHO cells that the proportion of mevalonate derived products incorporated into proteins is markedly dependent on mevalonate concentration (120). Furthermore, they reported the ratio of geranylgeranylated to farnesylated proteins was also dependent upon the concentration of mevalonate, with a linear increase in GG/F ratios with increasing exogenous mevalonate up to a ratio of 10 at 18 μM mevalonate. The studies with testicular cells also show dependence of incorporation of isoprenoids into proteins and the ratio of geranylgeranylation to farnesylation (Fig. 3-7 and Fig. 3-8) on exogenous mevalonate concentration with a ratio of about 3 at 11 μM mevalonate. The concentrations of [^{3}H]-mevalonic acid routinely used in these studies, 1.6 μM and 2.86 μM, permitted high levels of protein labelling, but variations in GG/F ratios between series of experiments limits comparison of different sets of data. Interpretations of GG/F ratios are based on their relative differences between ages and cell types in the same series of experiments.

In contrast to the similar levels of PFT and PGGT-I activity observed between pachytene spermatocytes and round spermatids, the ratios of geranylgeranylated to farnesylated proteins are reproducibly quite different. Fig. 3-10 and Table 3-3 indicate a 4-fold difference in GG/F ratios

between pachytene spermatocyte proteins (mostly geranylgeranylated, GG/F = 3.3 ± 0.3) and round spermatid proteins (highly farnesylated, GG/F = 0.8 ± 0.1). The difference appears to be caused more by a reduced amount of geranylgeranyl/mg protein in the round spermatids since the amount of farnesyl/mg protein is similar in both the round spermatids and the pachytene spermatocytes. The whole spermatogenic cell population from the same adult animal, which has a high proportion of pachytene spermatocytes and round spermatids, gave a ratio of 1.9 ± 0.3. Evidently, the levels of PFT and PGGT-I activities are not indicative of the types of polyprenols associated with the proteins between these two cell types. The differences in ratios may represent differences in the types of protein substrates synthesized between the two cell types. (There is always the possibility that these ratios may not represent actual proportions of GG/F in the animal due to different in vivo mevalonic acid concentrations.)

Age-dependence of in vivo protein prenylation. Age dependent differences in the level of prenylation among individual and groups of proteins were explored by analysis of 1-D and 2-D gel electrophoretograms of [^{3}H]-mevalonic acid labelled proteins from rat seminiferous epithelium. The most striking difference from 1-D gels (Fig. 3-12) showed a 2-fold decrease in the total amount of radiolabel per mg protein from 9 to 17 days of age with a slower decline from

17 to 105 days of age. The decline in protein prenylation with age may reflect a dilution of cells with high prenylation capacity, which predominate early during spermatogenesis, by the appearance of more mature spermatogenic cells, less active in prenylation. This phenomenon is observed with other enzymatic activities in the developing rat testis (126, 127). However, this does not explain the sudden drop in protein prenylation by day 17, a period before which large scale changes in differentiation occur. Since the endogenous pool of mevalonate is depleted with mevinolin before and during [^{3}H]-mevalonic acid labelling, the decline should not reflect a change in pool size. Changes in the pool size of mevalonate products, including farnesyl diphosphate, due to variations in enzyme activities along this metabolic pathway could affect protein prenylation. However, incorporation of [^{14}C]-acetate into dolichol and cholesterol, which are also synthesized with farnesyl diphosphate, have been shown to increase with succeeding prepuberal stages to give high levels in adult testes (23). Also, the decrease in labelling is not due to a decrease in [^{3}H]-mevalonic acid uptake into the cells, since these studies show similar levels of labelling of cholesterol and dolichol at 9 ,23 and 35 days (Fig.3-13). The decrease in prenylated proteins may reflect a decrease in synthesis of available protein acceptors at 17 days.

Another age-dependent difference in protein prenylation is the increase of label in 44 kDa protein(s) (22.8%) relative to the 20-30 kDa proteins at 23 days of age compared to lower levels (5.0 % and 5.8%) seen in 9 day old and 35 day old animals. Autoradiograms of 2-D gels containing [^{3}H]-mevalonic acid labelled proteins from the seminiferous epithelium of rats aged 9, 23 and 35 days shows a 44 kDa protein at 23 days with an acidic pI which is not evident at 9 or 35 days of age. The presence of multiple spots and streaking of the 44 kDa proteins over an extended pI range indicates that they may also be phosphorylated. Sepp-Lorenzino et al. observed that most prenylated proteins in murine lymphoma cells were also phosphorylated (118), and they described a [^{3}H]-mevalonic acid labelled 44 kDa protein as a nuclear matrix-intermediate filament protein. There are many implications for the involvement of such proteins in the meiotic events associated with cellular division occurring at this time of spermatogenesis. In addition, age-dependent changes in the levels of phosphorylation of such proteins could represent a mechanism for regulation of the function of the 44 kDa proteins.

Further analysis of autoradiographs of the 2-D gels shows a large increase in prenylation of a 36 kDa protein relative to the other proteins at 23 days. The mode of prenylation of the 44 kDa and 36 kDa proteins is primarily farnesylation (Fig. 3-5A,B), although the 36 kDa protein

contains some geranylgeranyl as well. The increase of farnesylation of these proteins occurs coordinately with the increase of PFT activity. Although the overall level of protein farnesylation does not appear to change, the increase in PFT activity may be relevant for a particular protein or proteins at 23 days.

Autoradiographs of both 1-D and 2-D gels show the label in the 20-30 kDa molecular weight region decreases dramatically from 9 to 17 days. This is not surprising since most of the labelled protein migrates to the 20-30 kDa region, which would reflect the effects of overall prenylation. As mentioned above, many of the prenylated proteins of this region are members of the ras family of gene products that function in signalling pathways and in vesicular traffic. The potential roles of these proteins in early events of spermatogenesis in prepuberal rats are numerous. The labelling of these 20-30 kDa proteins at 9 days of age is the highest of all the ages studied. Nine days signifies the onset of meiosis, when the preleptotene spermatocytes first appear and replicate their DNA (128). The protooncogenes c-myc, c-fos and c-jun whose gene products function as transcriptional activators (129-131) are expressed at high levels in preleptotene spermatocytes (12). It has been suggested that as these cells complete the last mitotic division and enter prophase of meiosis, the nuclear proto-oncogenes function in the alteration of gene

expression (12). It is known that the developing germ cells directly affect the activity of the Sertoli cells (132). Therefore, one might predict this time frame to represent marked interdependence between germ cells and Sertoli cells. The involvement of low molecular weight G-proteins in signal transduction following cellular response to growth factors as well as vesicular transport and secretion may be particularly important in 9 day old seminiferous epithelium.

Spermatogenic cell type differences in in vivo protein prenylation. In vivo differences in protein prenylation between pachytene spermatocytes and round spermatids are very striking. One-dimensional gel electrophoresis reveals a significant lack of prenylation of the 44 kDa protein in pachytene spermatocytes relative to the whole germ cell population, whereas the 20-30 kDa regions are similar in the levels of prenylated proteins. Two-dimensional gel electrophoresis displays the 44 kDa protein(s) preferentially labelled in the round spermatids with low levels of labelling in the 20-30 kDa region. These results support the finding that geranylgeranylated proteins outnumbered farnesylated proteins 4/1 in pachytene spermatocytes compared to round spermatids, since the 20-30 kDa proteins are predominately geranylgeranylated.

The round spermatids display a GG/F ratio of <1 which reflects the low levels of label in the 20-30 kDa proteins and the relatively high levels of farnesyl label in the 44

kDa protein(s). The haploid round spermatids undergo a series of transformations in the process of spermiogenesis including extensive morphological changes in which the spermatid elongates and the acrosome is formed (135). The acrosome forms at the Golgi complex which fuses with the nuclear envelope, then the nucleus and acrosome system locate to the periphery of the cytoplasm. Further changes include changes in chromatin and in nuclear proteins. At spermiation, the cytoplasm separates from the spermatid to form the mature spermatozoa. Undoubtedly, a nuclear matrix-intermediate filament protein would be actively involved in all of these transformations. It should be recalled that others have described a prenylated 44 kDa protein among the nuclear matrix-intermediated filament proteins (127). Other work also relates cell morphology changes with isoprenoid metabolism. Blocking of mevalonate synthesis in proliferating cell with lovastatin has profound consequences for maintaining cell shape (133), and microinjection of a competitive inhibitor of protein prenyltransferases into the cytoplasm of cells induces the same change in morphology with loss of actin cables (134). All of these data indicate that prenylated proteins have specific roles in the meiotic events as well as differentiative events of spermatogenesis.

CHAPTER IV
CONCLUSIONS AND FUTURE DIRECTION

The results described in Chapters II and III provide evidence of distinct age and cell type differences in protein prenylation in rat seminiferous epithelium. These differences are observed in the protein prenyl transferase activities, in the level and mode of prenylation, and in the types of proteins prenylated. The most striking changes were observed at two distinct ages of the prepuberal rat which correspond to specific stages of spermatogenesis since the prepuberal period represents the first time newly differentiated cells emerge during spermatogenesis. These stages represent critical events in spermatogenesis for the continuation of the developmental process. At this point in time techniques are not available for inhibiting protein prenylation in the testes of a live animal in order to examine any effects on the normal progression of spermatogenesis. However, the observed cell- and age-dependent changes in protein prenylation appear to have a substantial impact on the events of spermatogenesis in the developing testes.

The levels of PFT and PGGT-I were observed to increase 2- to 3-fold in whole testes, seminiferous epithelium and spermatogenic cells at 23 days of age as described in Chapter II. The low levels of PGGT-II appear to be unchanged at this time. Indirect evidence suggests that the secondary spermatocyte is responsible for the peak in activity. This cell is derived by the first meiotic division of the pachytene spermatocytes which predominate at 23 days of age. The secondary spermatocyte is short-lived before dividing in the second meiotic division to form the haploid round spermatids. Although 23 day old spermatogenic cells, and most likely the secondary spermatocyte, have high PFT activity, in vivo labelling studies indicate little if any change in the level of total protein farnesylation. This occurs despite an increase in prenylation of the 36 kDa and 44 kDa proteins in seminiferous epithelium at 23 days of age and the apparent absence of 44 kDa proteins, which are farnesylated, in the pachytene spermatocytes. As the spermatocytes divide into round spermatids, the labelling of the 44 kDa protein remains high relative to total protein prenylation, despite lower PFT activity in round spermatids.

It appears that the protein substrates available for prenylation change (in the round spermatid) irrespective of enzyme activity. PFT and PGGT-I activities may be increased at 23 days for the purpose of prenylating particular proteins such as the 36 kDa and 44 kDa proteins. These

proteins may play an important role in the meiotic divisions of the secondary spermatocyte. In HepG2 cells, a 44 kDa prenylated protein was co-localized with the nuclear matrix and intermediate filaments (118). It could be surmised that the 44 kDa proteins function in organization of the nucleus for sustaining the events of the meiotic divisions. Continued high levels of farnesylation of the 44 kDa proteins in the round spermatids indicate that these proteins also play a role in the events of spermiogenesis, as described in the discussion section of Chapter III. The morphological transformations of spermiogenesis involve formation of the acrosome, relocation of the nucleus and acrosome system, transformation of nuclear proteins and chromatin, and elongation of the spermatid (132). Interesting to note is the appearance of low pI 44 kDa protein(s) at 23 days. This could represent an increase in phosphorylation levels of other 44 kDa proteins which would imply precise regulation of the protein at 23 days, coincident with increased PFT activity.

Other changes in protein prenylation are the levels and the mode of prenylation at 9 days of age, described in Chapter III. There is a 1.5-2.0 fold decrease in the level of total protein prenylation from 9 to 23 days of age and the same decrease in the level in geranylgeranylation with little change in farnesylation. (A slow decline of both geranylgeranylation and farnesylation was also observed with

age in the adult rat, possibly due to the accumulation of mature, less active cell types.) Most of the geranylgeranylated proteins migrate as 20-30 kDa proteins; similar findings have been reported in other cell types. Somewhat contradictory are the observations that the high levels of prenylation of these proteins at 9 days are accompanied by low levels of PFT, PGGT-I and PGGT-II activities. These results again lead to the conclusion that in vivo changes in protein prenylation are apparently due to changes in protein substrate availability in spermatogenesis, rather than enzyme activity levels.

Many of the low molecular weight prenylated proteins are members of the ras family of genes that function in second messenger activity and membrane trafficking. As mentioned, these activities are particularly important for 9 day old seminiferous epithelium which is marked by the entry of spermatogenic cells into prophase of meiosis. The spermatocyte has just completed the last mitotic division and undergone DNA synthesis. The Sertoli cells are actively involved in protein synthesis and secretion in response to the spermatogenic cells and to hormonal stimuli. Cell signalling in response to proliferative stimuli and vesicle transport in the production and secretion of proteins are roles that the $p21^{ras}$-like proteins play in 9 day old seminiferous epithelium as meiosis begins. The function of the modification of proteins with isoprenoids has been

explored extensively. Initially, prenylation was thought to impart membrane localization for the protein (117). This is still believed to be true, yet the partitioning of prenylated proteins to many different intracellular membranes renders the localization process more complex. Several models have been suggested including a prenyl protein receptor in membranes, a lipid mediated non-specific affinity for membranes with specific targetting by protein-protein interactions, a lipid-lipid interaction, and lastly, a prenyl-dependent secondary modification that mediates protein targetting (135). The latter model also opens up possibilities for dynamic functions of prenylated proteins as suggested for palmitoylated and methylated proteins. Whereas the prenylation of proteins endures for the life-time of the protein, palmitoylation, methylation and phosphorylation may be reversed by more rapid turnover than the protein itself (136, 137). Palmitoylation and depalmitoylation are independent dynamic processes for the function of rhodopsin (138), and transient methylation of farnesylated lamin B is believed to play a role in the disassembly of the nucleus during mitosis (137). Reversible phosphorylation of prenylated proteins has also been reported. Rap1b is involved in intracellular signalling and phosphorylation has been found necessary for interaction between the prenylated carboxyl terminus of Rap1b and the GDP exchange factor GDS (139). $p21^{ras}$ proteins are

phosphorylated on specific serine residues (140) and methylated on the carboxyl terminus (136). These reversible modifications of the prenylated protein have been proposed for the specific localization of the protein and the regulation of the activity of the protein (135). The isoprenoid modification of proteins is a prerequisite for further modifications and it functions to promote protein interaction with cellular membranes and as a recognition site for interaction with cytosolic proteins. For example, the isoprenylation-dependent reversible association of rab G-proteins with membranes has been shown to play a crucial role in membrane trafficking (141). This explains the multiple subcellular location of prenylated proteins and provides a model for the functions of these dynamic proteins.

Several groups have observed arrest of the cell cycle caused by the depletion of non-sterol isoprenoids in animal cells (30, 36, 46). Studies have pursued the possibility that the responsible mevalonate derived non-sterol product is(are) prenylated protein(s). Sepp-Lorenzino et al. report two distinct cell-cycle-phase-dependent patterns of prenylation in synchronized HepG2 cells. They found increased production of prenylated GTP-binding proteins of 21-24 kDa during mid-to-late G1, presumably for the development of responsiveness towards proliferative stimuli (93, 124). In contrast, there was an increase in prenylation

of 44 kDa and 69 kDa nuclear matrix-intermediate filament proteins during late G1/S, implying a role for these proteins in maturation of the nuclear matrix for DNA replication (93). Changes in protein prenylation patterns have also been shown to occur during early stages of spermatogenesis. Nine-day-old seminiferous epithelium is also particularly responsive to proliferative stimuli and is characterized by the increased production of prenylated 20-30 kDa proteins. While a distinct increase in prenylation of the 44 kDa proteins at 9 days, when DNA replication occurs, was not discerned in this work, an increase was observed at 23 days time when the meiotic divisions occur. In the literature, much emphasis has been placed on the role of protein prenylation in cell growth and proliferation. This work provides evidence of the involvement of protein prenylation in the developmental growth process of spermatogenesis. The in vivo and in vitro analysis of protein prenylation during spermatogenesis has revealed remarkable similarities and differences with that observed by others in mitotically dividing cells. Since inhibition of protein prenylation appears to inhibit cell cycling, it could be predicted that the mitotic and meiotic divisions of spermatogenesis would also cease. The results presented here warrant further studies for more specific analysis of the effect of protein prenylation on spermatogenesis. The rat seminiferous epithelium provides a unique model that allows

examination of distinct developmental stages for elucidating the role of protein prenylation in a dividing cell.

Future directions for studying protein prenylation in spermatogenesis should focus on the changes at 23 days of age in the seminiferous epithelium. Studies in the live animal would be difficult to design. Attempts to inhibit protein prenylation would necessitate large doses of mevinolin that would incur many side effects, including death. Therefore, future studies would concentrate on isolated cells. Cultures of 23-day-old seminiferous epithelium could be treated with the hormones FSH, insulin, testosterone and retinol for analysis of effects on protein prenylation. Other biolological effects have been shown to occur with the addition of these hormones, such as increased secretion of transferrin (20).

On a more molecular level, measurements of spermatogenesis-age-dependent levels the PFT and PGGT-I α and ß subunit mRNAs would indicate whether transcriptional regulation is responsible for increased activity at 23 days. High levels of PFT α subunit mRNA have been found in the testes (73) but only low levels of ß subunit have been described (74). These findings are unexpected since the protein α/ß subunits exist as 1:1 heterodimers. The PFT α subunit is believed to be the same protein as the PGGT-I α subunit (75) and this subunit may confer catalytic function for the enzymes (142). Since separate but homologous ß

subunits make up the structures of PFT and PGGT-I, they presumably confer substrate specificity (75). A possible explanation for the disparate levels of α/ß subunits may arise from yeast genetic studies that indicate multiple yeast genes that encode PGGT-I α subunits (135). A model has been proposed that suggested that sharing of ß subunits may allow divergent prenyltransferase specificities, whereas sharing of α subunits may generate multiple prenyltransferase isozymes with divergent regulatory properties (135). Regulation of cellular levels of these individual subunits may play a larger role in specific age-dependent protein prenylation. The difference in the expression levels of these subunits between pachytene spermatocytes and round spermatids could also shed light on the regulation of specific protein prenylation, where the levels of PFT/PGGT-I activity between pachytene spermatocytes and round spermatids is not that different. The existence of a unique transcript of FPP synthase in the haploid spermatids (13) promotes particular interest in analyzing the potential regulation of the protein prenyl transferases, which utilize FPP, in these cells. The capacity for round spermatids to synthesize high levels of FPP is consistent with the relatively high levels of protein farnesylation. Yet PFT and PGGT-I activities have declined to low levels by 25 days of age (when round spermatids first appear). This suggests that alternative branches of the

mevalonate pathway are also active. HMG CoA reductase activity and cholesterol synthesis are low in mouse spermatids, yet dolichol synthesis is high (23), although the data in Chapter II show somewhat decreased levels of dolichol synthesis in the rat round spermatids. Nonetheless, the unusually high levels of farnesylation versus geranylgeranylation in these cells suggest very different and possibly unique levels of PFT and PGGT-I transcripts.

Further characterization of the prenylated proteins of the seminiferous epithelium is also of interest. In particular, the 44 kDa protein changes with respect to prenylation and possibly phosphorylation in 23 day old rats, while the 36 kDa protein also changes in levels of prenylation in 23 day old seminiferous epithelium. The 36 kDa is particularly interesting because it appears that both geranylgeranyl and farnesyl are incorporated into the protein. This protein may contain unique sequences for recognition by prenylating enzymes. Sequence information of these proteins could be obtained for possible correlation with other proteins (e.g. nuclear matrix-intermediate filament type proteins). Antibodies could also be made to the proteins for examining potential changes in protein levels with spermatogenesis.

Finally, a new approach for identifying other PGGTs may be applied using the technique described in Appendix B. Soluble, unprocessed proteins can be collected from

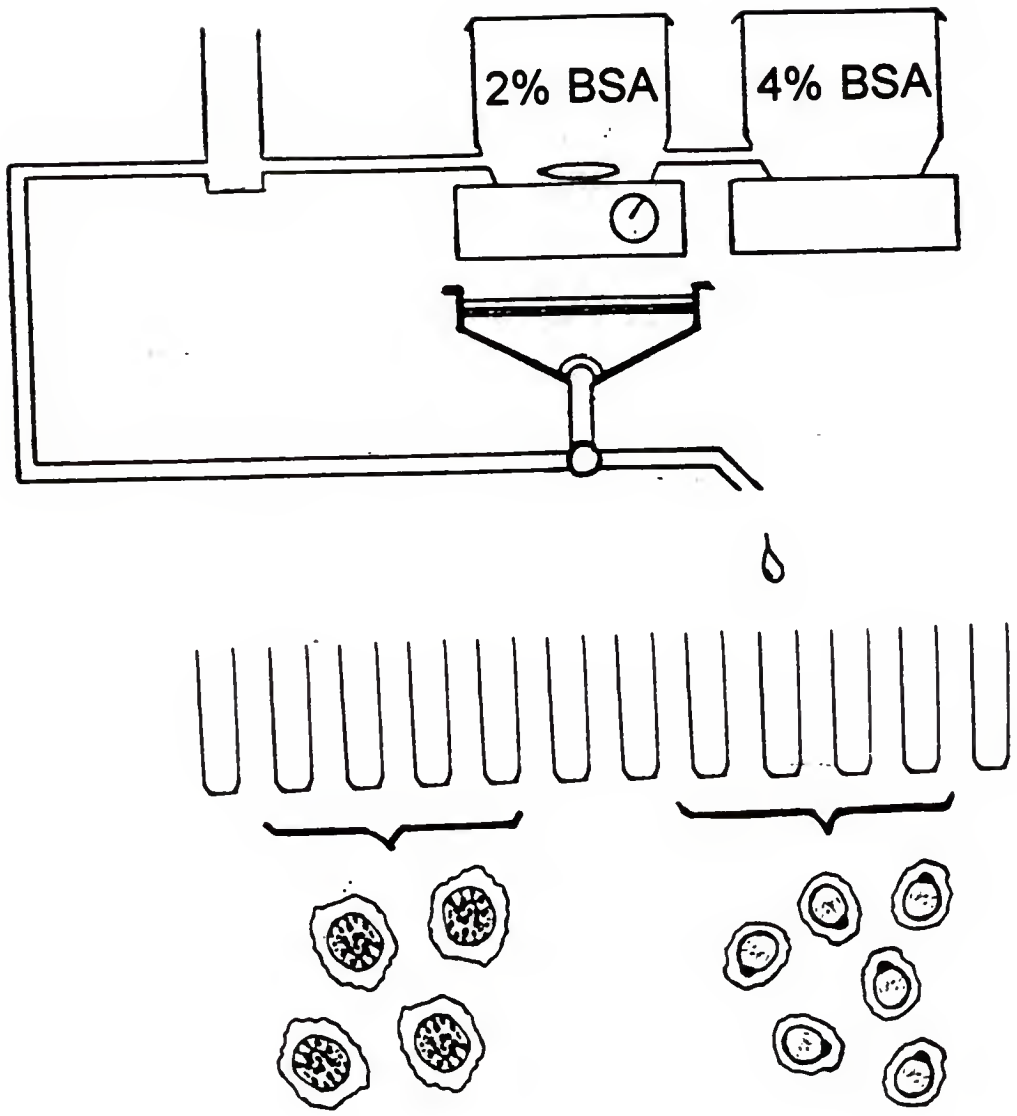
mevinolin treated 9 day old testicular cells for use as substrates for PGGT assays. Cytosols subjected to chromatographic separation would provide fractions that can be assayed for PGGT using the unprocessed endogenous proteins as substrates with [^{3}H]-GGPP. N-acetylated peptides could be used as inhibitors to distinguish new PGGTs from PGGT-I activity.

These analyses would aid in the delineation of the type of regulation involved in the changes of protein prenylation observed at 9 and 23 days in the seminiferous epithelium. These cells undergo distinct spermatogenesis-age-dependent protein prenylation changes and provide an excellent system for studying developmental regulation of protein prenylation. Reported studies on protein prenylation have focused on the involvement of prenylated proteins, especially p21^{ras}, on the cell cycle. The evidence reported here indicates a strong involvement of prenylated proteins in the development of the germ cell. In order to properly understand the role of protein prenylation in a cancerous cell or immortal cell line, the mechanism of protein prenylation in a normal differentiating cell must be clarified.

APPENDIX A
DIAGRAM OF STA PUT CELL SEPARATION PROCEDURE

Figure A. Diagram of Sta Put Cell Separation Procedure

Spermatogenic cells were isolated from rat testes and separated by unit gravity in the Sta Put separation procedure described in the methods of Chapter I.


2% BSA
4% BSA

APPENDIX B
IN VITRO PRENYLATION OF ENDOGENOUS SEMINIFEROUS EPITHELIAL PROTEINS SUPPORTS DATA IN CHAPTER III

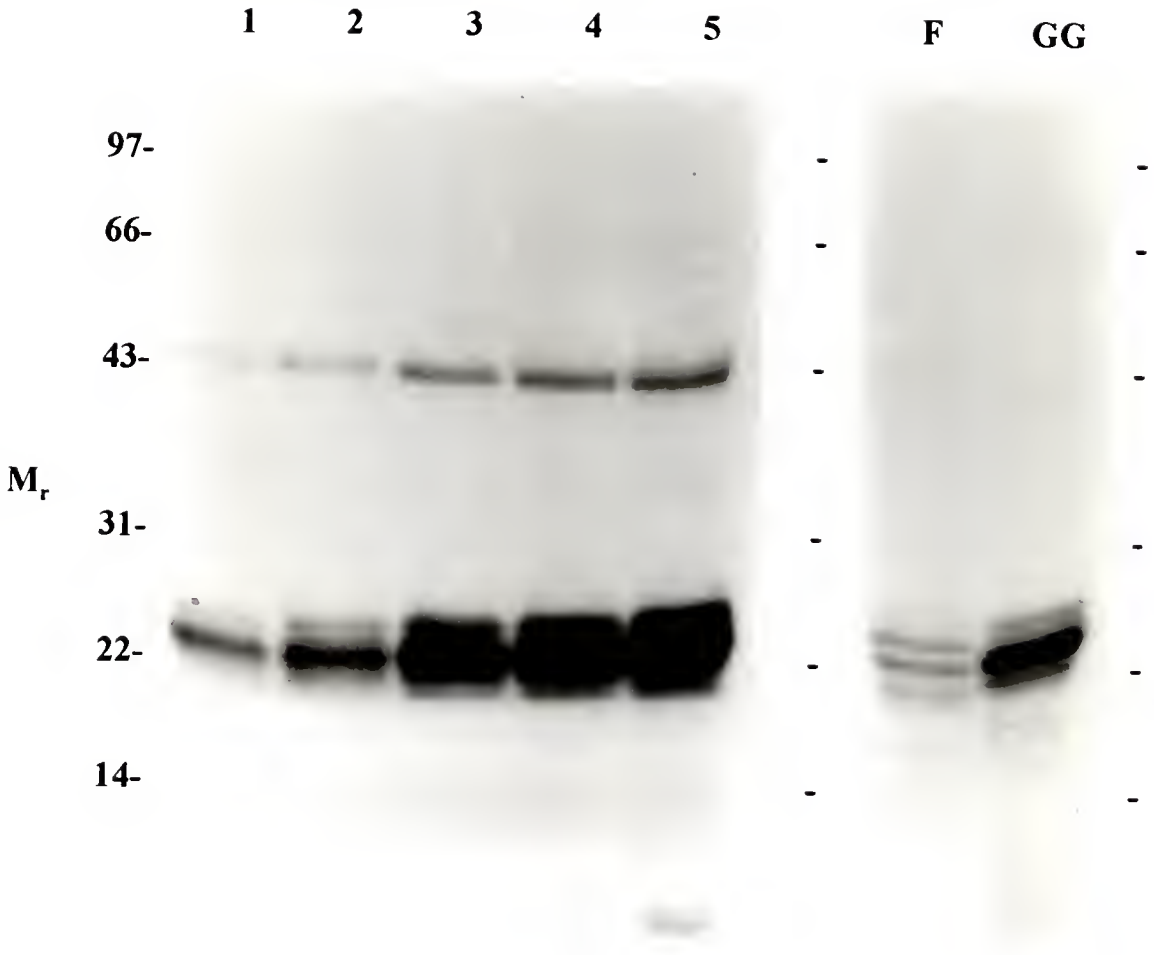
In further attempts to distinguish which [^{3}H]-mevalonic acid labelled proteins are farnesylated and which are geranylgeranylated in rat seminiferous epithelium, cytosols from mevinolin treated cells were assayed for [^{3}H]-FPP or [^{3}H]-GGPP incorporation into the accumulated apoproteins. The seminiferous epithelium was isolated from 37 day old animals and treated with mevinolin for 6 h. Soluble endogenous proteins were isolated from the cells and incubated with [^{3}H]-FPP or [^{3}H]-GGPP as described in the legend of Fig. A. As a control, the same cells were labelled with [^{3}H]-mevalonic acid as described in the methods of Chapter III.

SDS-PAGE and autoradiography reveal that the proteins labelled with [^{3}H]-mevalonic acid are also labelled with either [^{3}H]-FPP or [^{3}H]-GGPP. The data supports the results shown in Fig. 3-5 which identifies the type of polyprenol attached to proteins (labelled in vivo with [^{3}H]-mevalonic acid) in particular molecular weight regions of SDS-PAGE. Consistent with the previous data, the proteins in the 20-30 kDa molecular weight region are predominately

geranylgeranylated with some farnesylated proteins evident. Also consistent with previous data is the apparent farnesylation of the 36 kDa protein, yet it is difficult to identify if it is also geranylgeranylated. A 44 kDa protein is farnesylated and a lower, previously unidentified band appears to be geranylgeranylated. Fig. A also indicates that a 56 kDa protein is farnesylated and a 67 kDa protein is geranylgeranylated.

Figure B. Electrophoretic Profile of Farnesylated and Geranylgeranylated Proteins in the Seminiferous Epithelium

Seminiferous epithelium was isolated from a 37 day old rat and treated with 30 μM mevinolin for 3 h, followed by 3 h of incubation with 30 μM mevinolin with (1-5) or without (F, GG) [^{3}H]-mevalonic acid as described in the methods. [^{3}H]-Mevalonate labelled proteins were subjected to SDS-PAGE (lane 1: 65 μg, 21,600 dpm; lane 2: 130 μg, 43,300 dpm; lane 3: 260 μg, 86,600 dpm; lane 4: 390 μg, 129,900 dpm; lane 5: 520 μg, 173,100 dpm) and autoradiography for 20 days. Unlabelled cells were lysed in 50 mM Tris-HCl, pH 7.0, 10mM dithiothreitol, 10 mM $MgCl_2$, 1mM PMSF. The supernatant (50 μl) remaining after centrifugation at 100,000 x g for 30 min was incubated for 1 h at 37°C with 2 μCi (2.5 μM) [^{3}H]-FPP (F) or 2 μCi (2.5 μM) [^{3}H]-GGPP (GG). Acetone (1 ml) precipitates were subjected to SDS-PAGE and autoradiography for 20 days. The migration of molecular weight standards (x 10^{-3}) are shown.


1
2
3
4
5
F
GG
97-
66-
43-
M_r
31-
22-
14-

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Jan Dugan was born in Evanston, Illinois, in 1965. In 1983, she started her work on her B.S. in biology at Radford University in Virginia. This was completed in 1987, when she began her graduate studies in biochemistry and molecular biology at the University of Florida. After working for nearly two years in the laboratory of Dr. Michael Kilberg, she began her thesis work in the laboratory of Dr. Charles Allen.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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